Jarabek 44994

DOSE RESPONSE CHARACTERISTICS OF UTERINE RESPONSES IN RATS EXPOSED TO ESTROGEN AGONISTS

Hugh A. Barton*, Melvin E. Andersen, Bruce Allen

In Press: Reg. Tox. Pharm.

*To whom correspondence should be addressed at:
K.S. Crump Group, ICF Kaiser
PO Box 14348
3200 Chapel Hill Nelson Hwy, Suite 101
Research Triangle Park, NC 27709
(919) 547-1709
(919)547-1710 FAX
habarton@aol.com

July 3, 1998

Short Title: Dose Response Characteristics of Uterine Responses

ABSTRACT

Assays for uterine response have played major roles in developing an understanding of estrogen-mediated processes and for identifying compounds with hormonal activity. Data from assays measuring increases in uterine wet weight in rats were evaluated in terms of their dose response characteristics. Analysis using a Hill equation found inconsistent estimates for the ED_{50} (concentration giving half maximal response) and n (steepness of response) among the assays. This variability reflects disparate assay protocols and limitations of the dose response data collected in the experiments. Although uterine wet weight is easily measured, it arises from several physiological processes (e.g. water retention, cell proliferation). This contributes to the assay variability with different protocols. The potential use of the Hill equation for dose response analysis to estimate a benchmark dose was also considered using these data sets as surrogates for receptor-mediated toxicological effects. Strengths and weaknesses were identified, but overall the Hill equation should likely become a favored option for determining a benchmark dose, particularly when a data set demonstrates a maximal response. For screening purposes, empirical analysis using the Hill equation provides adequate information for classifying and prioritizing compounds. To develop an understanding of how incremental exposures to compounds with estrogen agonist activities would affect intact adult females, quantitative analyses are required that account for the pharmacokinetics of estradiol and subsequent interactions of the receptor complexes in regulating the responses.

INTRODUCTION

Uterine responses were among the first to be correlated with estrogen agonist activity and have continued to be commonly used for identifying estrogen agonists (Clark and Peck, 1979). Uterine assays have been used predominantly by the pharmaceutical industry to evaluate estrogen agonist activity of potential therapeutic compounds. Recently these assays have been included in several proposals for screening endocrine active compounds (EACs) for purposes of identifying environmental compounds with some potential to cause toxicity by disrupting endocrine function (O'Connor *et al.*, 1996; Shelby *et al.*, 1996; Odum *et al.*, 1997).

The most commonly measured uterine response is a change in wet tissue weight, though endpoints that also have been measured range from responses in whole tissue or cells to subcellular biochemistry (Table 1). Increased weight and most of the other uterine responses studied are not themselves adverse endpoints. Rather they are of interest as well studied examples of estrogen receptor-mediated effects that provide insight into issues that likely will arise for receptor-mediated toxicity due to underlying similarities in hormonal signaling processes. Alterations in uterine weight, while simple to measure in the laboratory, do not represent a simple or even just a single response. Rather, observed uterine weight reflects a variety of processes including alterations in vascular permeability, water retention, cell size, and cell number due to proliferation (Clark and Peck, 1979; Reel *et al.*, 1996).

Uterine responses have been measured in several strains of rats and mice under a variety of assay conditions (Table 2). Immature animals (prior to initiation of estrus cycling) and ovariectomized adult animals have been tested. Exposure routes have included oral, subcutaneous injection, intraperitoneal injection and silastic implants with several vehicles. Dosing frequency and duration (doses per day and total number of days dosed) as well as the time post-dosing of uterine examination have varied as well. These variations can significantly impact the results of the uterine assays as observed with estriol, for example; assays using different methods showed positive or negative results (Clark and Markaverich, 1983).

The predominant pharmaceutical applications of uterine assays have focused on classifying compounds as potential estrogen agonists and estimating their potency and

efficacy relative to estradiol (E2) (Reel *et al.*, 1996). Analysis of the results often involves log transformation of the data and fitting it with a straight-line equation in order to estimate the dose associated with half-maximal response, ED_{50}. Limited consideration has been given to development of quantitative information necessary for prioritization of compounds for further testing or for dose response analysis for use in risk assessment. The planned, widespread use of this assay argues for standardization of test protocols and more complete evaluation of the dose response relationships. These dose response analysis methods will be applicable to a range of other assays involving endocrine-mediated effects, including *in vitro* assays for receptor-mediated gene activation (Zacharewski, 1997).

This report characterizes the uterine assay in the context of its potential use for prioritization of compounds. Implications for dose response analysis and risk assessment of endocrine-mediated toxicities were drawn, assuming that these toxicities would show similar dose response characteristics as the uterine responses which are not generally considered adverse effects. It explores the use of the Hill equation for data analysis and the impact of variations in assay protocol on the results. Issues associated with potential application of the Hill equation for benchmark dose (BMD) approaches are described. The results of the analysis suggest that experimental design and BMD definition issues need to be resolved. Appropriate consideration of dosimetry (pharmacokinetics) and mode of action (pharmacodynamics) will be useful for this assay to reach its full potential in the context of EACs screening and risk assessment.

METHODS

Dose Response Modeling:

For this analysis, a specific dose response model, in the form of a Hill equation, was fit to data on uterine wet weight following exposures to E2 or estriol. The Hill equation is expressed as:

$$m(d) = m(0) + Dm * d^n / (ED_{50}^n + d^n)$$

where m(d) is the mean uterine wet weight for dose d, and the parameters m(0) (the mean wet weight in the absence of exposure), Dm (the maximal increase over baseline), ED_{50} (the dose giving a response 50% of maximal), and n (often referred to as the Hill coefficient) were estimated by maximum likelihood techniques (Barlow 1980). Normal variation around the mean weight was assumed, in part, reflecting the data that were reported as mean values and either standard deviations or standard errors; the degree of variation was allowed to vary across dose groups. The parameter n was constrained to be greater than or equal to zero (i.e. a positive value). All dose terms were expressed in terms of μg compound per kg body weight. The maximum likelihood fitting routine was implemented using the solver capabilities in EXCEL (Microsoft, Redmond, WA). Maximum likelihood estimates are those most likely to have given rise to the observed data, if the Hill equation does in fact represent the appropriate form of the dose response relationship.

Data Sets:

Data sets selected for analysis were published results of assays using rats dosed with estradiol (E) or estriol that satisfied the following criteria:

- it was possible to estimate exposure doses in units of μg/kg;
- sample sizes for each dose group were given (or, at least a relatively small range of group-specific sample sizes could be inferred);
- mean wet weights and associated standard deviations could be calculated;
- at least four dose groups were tested and wet weight results were presented for one or more times after exposure.

The studies satisfying these criteria are listed in Tables 3 – 5 along with information about the assay method as reported by the authors.

In almost all cases considered here, the response data (means and standard deviations) were estimated from figures graphically depicting the uterine wet weight response as a function of exposure. The "digitizing" process that translated the graphical to numerical information is subject to error. Nevertheless, because this analysis focussed on evaluating issues (rather than formal dose response assessment of specific compounds, for example) the approximate nature of the input data was considered acceptable.

Modeling Outputs:

The results of the modeling that form the basis for the subsequent discussion consist of the maximum likelihood fits themselves (shown graphically) and the estimates of the parameters n and ED_{50}. No confidence limits for the parameters (or for possible BMD estimates) were calculated. Goodness of fit was determined by applying an F-test that evaluates the differences between observed and predicted responses, normalized by the observed, underlying variability.

RESULTS

For a wide variety of uterine weight assays, the Hill equation was found to fit the data quite well. Figures 1 - 4 typify the patterns of response and associated model fits for data obtained following E2 dosing. In only two of the cases examined were goodness of fit p-values less than 0.05, indicating some lack of consistency between observed means and model predictions (Table 6).

Modeling Effects of Assay Variations:

As noted in the introduction, the protocols that have been used for uterine assays vary with respect to species/strain of test animal, age, intact or ovariectomized, route of exposure, dose vehicle, and number of doses. The quantitative impacts of some of those differences can be seen in the variations in model fits, as shown here.

Somewhat consistent results can be observed for increases in uterine wet weight 24 hours after a single E2 injection as indicated by three data sets from separate laboratories (Anderson *et al.*, 1972; Kaye *et al.*, 1972; Grunert *et al.*, 1986). Each study used a fairly similar protocol with intact juvenile rats (see Table 3). The resulting model fits to these data sets differ some with respect to the predicted "location" and steepness of the dose response curve. The location of the curve is associated with the value of the parameter ED_{50}, greater values of that parameter indicating a curve shifted to the right in comparison to a curve with a smaller ED_{50} (all else being equal). Steepness is determined by the parameter n. Larger values of n are associated with curves that increase more rapidly in the region of the ED_{50} (i.e. from near-baseline levels to near-

maximal levels). Steeper curves also have a more discernible transition from dose regions for which increasing dose markedly increases weight, on the one hand, to regions for which increases in dose have little effect on weight, because near-maximal or near-baseline responses have occurred. Values for ED_{50} ranged between 0.3 and 0.8 μg/kg and values for n were 2.9, 1.3, and 1.0 in these three studies (Table 6). The difference in the steepness (transition from near-baseline to near-maximal response) is clear (Figure 1), while the similarity of the "location" of this increase is also apparent from the very similar values for ED_{50}.

Similarly, greater consistency in the ED_{50} as compared to the value of n was observed in the three studies using three daily injections of E2 followed by measurement of uterine weight 24 hours after the last dose (Lan and Katzenellenbogen, 1976; Kneifel *et al.*, 1982; Odum *et al.*, 1997). Values for ED_{50} were between 1.4 and 3.1 μg/kg while n values ranged from 0.5 to 6.0 (Table 6, Figure 2). The maximal increases in uterine weight are larger following three doses than a single dose, as are the ED_{50} values, though the significance of this latter observation is unclear.

Anderson and his colleagues also examined uterine wet weights at times other than 24 hours post-dosing (Anderson *et al.*, 1973; Anderson *et al.*, 1975). At 3 hours after the single E2 injection dose, the response curve was less steep than at 24 hours (Table 6, Figure 3); estimates of n for the two sets of 3-hour measurements were 1.6 and 1.1. At 6 hours after exposure, the curve was much steeper (n=3.4) than at 3 h, though the data of Grunert et al. (1986) at 6 hours do not support this (n=0.47). The ED_{50} values for the two sets of 3-hour measurements (2.2. and 2.6 μg/kg) were similar to one another and greater than the ED_{50} estimates for the 6-hour and 24-hour measurements. These differences reflect the time courses for the underlying biological processes; water retention has a maximum around 6 hours while cell proliferation occurs with a maximum around 24 hours. Notably, the cell proliferation at 24 hours reflects receptor occupancy at earlier times (around 6 hours post-dosing) rather than concurrent occupancy (Clark and Peck, 1979; Clark and Mani, 1994).

Dramatic differences associated with route of exposure have also been observed. Odum et al. (1997) exposed immature rats to E2 via subcutaneous injection

(in oil) or via gavage, for three days. The doses were 0.5, 1, 2, 5, 10, 20, 40, 200, and 400 μg/kg for the subcutaneous injections while the gavage study used doses of 10, 20, 40, 100, 200 and 400 μg/kg. The observed and model-predicted uterine wet weights (24 hours after the last dosing) are shown in Figures 2C and 4. The plateau of response is clear for the highest 6 subcutaneous doses tested, but no clear demonstration of maximal response was apparent for the oral exposures. The resulting differences in parameter estimates and curve shapes are striking. For the subcutaneous exposures, n=6.0 and ED_{50}=3.1 μg /kg, whereas for the oral exposures n=1.6 and ED_{50}=280 μg /kg. This latter ED_{50} estimate reflects the lack of a defined maximal response in the data resulting in a model predicted maximal response (wet weight of 138 mg) higher than any response observed in this experiment. The model-predicted maximal uterine response may be unrealistic, but if the maximal increase is restricted using the predicted maximal increase from the subcutaneous study, a poor fit is obtained with the oral data. The difference in the ED_{50} values reflects the markedly different pharmacokinetics of E2 given by these two dose routes (Longcope *et al.*, 1985; Lobo and Cassidenti, 1992).

Data Set Limitations and Parameter Estimates:

The Hill equation used in this analysis assumes some maximal response (i.e., the parameter Dm) constraining the true mean response for all exposure levels. Not surprisingly, then, the predicted dose response curves are sensitive to the presence or absence of a well-characterized maximal response in the data set being modeled.

The data of Odum et al. (1997) for the oral study (Figure 4) are a case in point. Because the evidence of a maximal response is limited at best, the maximum likelihood fit predicts a very large ED_{50} of 280 μg/kg and an n of 1.6. In extreme cases, as exemplified by the uterine weight responses after repeated subcutaneous estriol exposure (data of Lan and Katzenellenbogen 1976; Figure 5A), the data display only upward curvature with respect to uterine wet at the tested doses. Resulting ED_{50} estimates are extremely large (79000 μg/kg in the case of Figure 5A) and the predicted maximal response is biologically unrealistic (200 g for the data in Figure 5A). Without additional data or reasonable assumptions about the maximal response, such modeling might be considered meaningless. In fact, Lan and Katzenellenbogen (1976) also

tested ethinyl estriol cyclopental ether under similar conditions and established an estimate for the maximal response (for their particular test animals under the conditions of their experiments) of 120-140 mg wet weight (compared to the corresponding maximal response of about 90 mg as shown in Figure 2B for E2). If 120 mg is assumed to be the maximum response for estriol induced uterine weight and the model is refit to the data , the resulting curve shape is minimally different (Figure 5B), but the ED_{50} estimate is a less unreasonable 350 μg/kg.

In addition to data on the maximal response, model determination benefits greatly from having data constraining the values of n. Data points around the ED_{50} dose, displaying intermediate levels of response, are very helpful in this regard. The data from Anderson et al. (1973) for the single E2 exposure and uterine weight responses 3 hours after exposure (Figure 3A) provide an excellent example. The data points corresponding to the 1 and 2 μg/kg doses define how steeply the curve must be between the near-baseline and near-maximal responses observed at the other doses. The data for the 6-hour uterine response from the same study (Figure 3B) lack such intermediate response data points. The value of n (estimated to be 3.4) in this case is constrained from below since lesser values of n would not allow for the increase in response from near-baseline to near-maximal responses between 0.2 and 1 μg/kg. The value of n could, however be somewhat larger, resulting in a steeper curve. Similar observations pertain to the data in Figure 2C.

DISCUSSION

The recent interest in endocrine-mediated toxicities has raised questions about effective hazard characterization and dose response assessment for EACs. Uterine responses continue to be widely used for evaluating compounds for estrogen receptor-mediated activity and have been proposed for screening to identify compounds with endocrine activity (Reel *et al.*, 1996; Shelby *et al.*, 1996; Odum *et al.*, 1997). Therefore, data from uterine response assays were reviewed for three purposes: to evaluate use of the Hill equation with receptor-mediated responses as an option for empirical dose response analyses, to evaluate some aspects of the use of this assay for screening

purposes, and to determine data needs for developing a biologically-based quantitative understanding of the dose response for uterine changes *in vivo*.

Use of the Hill Equation:

The Hill equation was originally used to model oxygen binding to hemoglobin and subsequently has been widely used as a model of receptor occupancy (Barlow, 1980). Though estrogen-mediated uterine responses are receptor-mediated, the relationship between receptor occupancy and response is not a simple one. Therefore, the Hill equation is used as a convenient empirical mathematical model, but no mechanistic interpretations should be inferred from estimated values of the equation parameters.

The Hill equation was used in this analysis because it possesses several useful characteristics for fitting experimental data: 1) it has a maximal response rate; 2) it has the flexibility to describe a variety of curve shapes; and 3) certain of its parameters (n and ED_{50}) are invariant to common transformations of the response data (e.g., expression of the weight responses as percentage of controls). It should be noted that other models proposed for modeling continuous endpoints such as a polynomial or Weibull model (when constrained to be monotonically increasing) cannot fit the sigmoidal shapes implied by the existence of a maximal response. Other options, as exemplified by the Hill equation, are clearly required when the existence of maximal responses is expected. Murrell et al. (1998) have argued in favor of use of the Hill equation for a range of continuous endpoints all associated with receptor binding and, therefore, presumed to have a demonstrable maximum response.

The existence of a maximal response might appear to be a reasonable assumption for effects such as uterine wet weight, but the experimental data are inconsistent. Several of the studies discussed above clearly demonstrated a plateau in uterine wet weight for the highest doses tested (see Figures 1A, 2C, 3B). The implications of model estimation in the absence of a clearly established plateau of response (See Figures 1B, 2A, 2B, 4, 5) are, however, among the major issues still to be resolved. As shown above (in relation to the discussion of the estriol data of Lan and Katzenellenbogen, 1976; Figure 5), a "reasonable" maximum could be imposed on the modeling (i.e. fixing the value of Dm), thereby constraining the model predictions.

Whether constraining the maximal response would alter estimates of lower bounds for use in a BMD analysis has yet to be determined. However, the maximum response varies due to experiment- and compound-specific pharmacokinetic and pharmacodynamic differences, so it is unclear if a "reasonable" maximum always can be determined.

The value of the parameter n is associated with a potentially important feature of the shape of the dose response curve. When n equals 1, the predicted curve is linear at low doses; for values less than 1, the curve is "supralinear" at low doses. Moreover, for $n \leq 1$, there is no inflection point indicating a transition from convex to concave curvature. When n is greater than 1, then low-dose sublinearity holds and the inflection point does exist. The expectation of low-dose linearity has been proposed in the new cancer risk assessment guidelines as the basis for deciding how acceptable exposures are to be determined, whether by linear extrapolation or by margin of exposure methods (EPA, 1996). Should such decision points be considered for EACs, the estimation of the parameter n might become critical.

Data Needs with the Hill Equation:

Specific suggestions can be made with respect to the type of data collection (experimental design) that would improve estimation of n and the other parameters of a Hill equation applied to uterine assay data. Clearly, there is a need for more than a control group and three positive dose groups. Such a design will seldom characterize all the features of a dose response curve with a maximal response and, potentially, a nonlinear low-dose shape. It is recommended that designs of uterine assays be able to characterize the following key features:

1) The maximal response. It is crucial that the maximal response, if it exists, be reasonably established. As discussed above (in relation to Figure 5), failure to do so may adversely affect the estimation of ED_{50} and n. Conversely, model-dependence on an erroneously estimated maximum could also bias estimates of those same parameters. Two or three dose groups may be required to establish the presence of and determine the value of the maximal response.

2) Region where responses are changing rapidly. This region lies on either side of ED_{50}. Determination of the steepness of the increase in response is directly related to estimation of the parameter n. Another two to three dose groups might be best for the characterization of this region.

3) Range of doses with near-baseline responses. Determination of this range helps to determine if an inflection point is required in the dose response curve, i.e., whether a linear or nonlinear curve can explain low-dose behavior. The estimation of both n and ED_{50} would benefit from two to three dose groups establishing the boundaries of this region.

When the goal of an assay is to establish a dose response pattern and estimate doses corresponding to specific response levels, as opposed to testing to determine whether group means are statistically similar or not (which is the basis for establishing a NOAEL), fewer animals per group can be used. In fact, designs with more groups and fewer animals per group (keeping the total number of animals constant) are preferable from a dose response modeling perspective. One important prerequisite for widespread application of uterine assays for screening potential EACs is a research effort specifically addressing the question of optimal experimental design. Such an effort might include a simulation study that considered number of dose groups, their placement (spacing), and group-specific sample sizes for optimal modeling of responses whose dose response behavior is described by a Hill equation. Such an effort would also need to consider the impact of compound specific variations in pharmacokinetics, which, as illustrated for E2 and estriol, have a major impact on the data obtained.

Such a simulation presupposes that other aspects of the uterine assays have been standardized. As suggested by the results above, variations in the timing of uterine measurement, number of exposures, and route of exposure (among others), can lead to differences in estimates of the dose response pattern. Ideally one first should determine the design most toxicologically relevant to widespread screening of potential EACs. Then the details related to numbers of dose groups and sample sizes can be developed.

Benchmark Dose Estimation:

One potential use of the Hill equation would be to estimate a BMD for use in dose response assessment. The uterine responses evaluated here are not adverse effects so they are unlikely to be used for this purpose, but these data sets may be considered representative of data for receptor-mediated adverse responses. Therefore, they were used for evaluating some of the issues of applying the Hill equation for a BMD analysis.

Traditionally, the reference point taken from experiments like the uterine assay has been the NOAEL. The limitations of the NOAEL are well known, and BMD estimates of one sort or another have been proposed (Crump, 1984; Barnes *et al.*, 1995; Crump, 1995; Murrell *et al.*, 1998). No specific BMD estimates have been calculated in this analysis, but we consider here some of the options that are available in the context of the uterine assays and Hill equation modeling.

Uterine wet weight is a continuous variable. Such variables do not lend themselves directly to the ideas of "additional risk" or "extra risk" that have been the basis of BMDs defined for quantal (incidence) type endpoints (Crump, 1984; Barnes *et al.*, 1995; EPA, 1995). Rather, with a continuous endpoint, the effect of exposure is to shift the value of mean response.

One direct way to define a BMD, then, would be to define it as the dose corresponding to some fixed change in the mean, either an absolute change or a change relative to the background mean (percent change) (Crump, 1984). In analyses of body weight changes, for example, the toxicological rule of thumb that a 10% drop in the mean weight is an indicator of effect can be used as the motivation for defining the BMD to be the dose associated with a predicted 10% change in body weight. Clearly, were one to define BMDs for uterine weights on this basis, some careful consideration would need to be given so as to ensure that the selected changes were biologically meaningful responses.

Options based on absolute or relative change ignore the amount of variability around the mean values. To the extent that such variability is related to what is considered "normal," those approaches ignore important information that might help

define what is toxicologically meaningful. Approaches to defining BMDs that take variability into account have been proposed and could be applied to the uterine wet weight responses. For fetal weight changes in developmental toxicity assays, Kavlock et al. (1995) (Kavlock *et al.*, 1995) proposed direct normalization of changes in mean responses by a standard deviation measure (e.g., the standard deviation of the control group). Crump (1995), elaborating on an idea presented by Gaylor and Slikker (1990), demonstrated how changes in the mean can be related to probabilities of "adverse response," where an adverse response is defined as being one in the tail of the distribution of responses. This approach has been referred to as a "hybrid" approach, because it models the changes in the means of a continuous variable but expresses BMDs in relation to probabilities of response as is done for a quantal (incidence) variable. Crump (1995) also showed that the approach using direct normalization by standard deviations and the hybrid approach are interchangeable.

The desirable features of the hybrid approach include the fact that BMDs are expressed in terms of extra or additional risk, providing a direct link to quantal endpoints, and that it requires toxicologists to define what is considered adverse or abnormal. In the context of uterine wet weight, however, some care would have to be given were this option to be pursued. Suppose, for example, that the maximal response was defined as the criterion distinguishing normal from abnormal responses. This is not an unreasonable choice; it should represent a high-end observation. If the model correctly predicts the maximal response, then the maximum predicted probability of response for an individual will always be less than 50%, no matter how large the dose may be. This is true because the model predicts that the maximal response is the limiting *mean* value.

The previous discussion does not imply that a risk near 50% would or should be considered in defining a BMD. But it does indicate that, no matter what the cutpoint, the probability of response does not increase to 100% as exposure levels increase, as would be expected of a quantal endpoint. The existence of a maximal mean response alters the relationship between quantal endpoints and continuous endpoints that has been one key element supporting the use of the hybrid approach. Nevertheless, with careful consideration of the implications of the existence of a maximal response, use of

the hybrid approach for uterine wet weight effects would have the advantage that the BMDs would be directly comparable to BMDs derived for continuous (and quantal) endpoints for other types of toxicity.

A third option for defining a BMD is based on the estimate of the maximal response. For example, the ED_{50} in the Hill equation predicts the dose at which the average response is half-way between the baseline and the maximal responses. Other ED_{100x} parameters are directly related to ED_{50} through the parameter n. In fact,

$$ED_{100x} = ED_{50} [x/(1-x)]^{1/n}$$

for x between 0 and 1 (e.g. for x = 0.1, $ED_{100x} = ED_{10} = 0.11ED_{50}$ for n=1.) As can be seen from this equation, other ED_{100x} values are sensitive to the steepness of the dose response curve represented by the value of n. Such sensitivity might recommend a value like ED_{10} rather than ED_{50} itself as the basis for the BMD estimate.

Use of ED_{100x} as the definition of the BMD would be different from the first two options, i.e. it is not a fixed change from baseline, nor does it correspond to a fixed probability of response. Selection of an ED_{100x} value as the definition of the BMD could be reasonable if a sound toxicological interpretation was associated with being some portion of the way between baseline and maximal mean responses. Such an interpretation would be unique to the uterine assay response and any other responses that eventually might be modeled with an equation having a maximal response such as the Hill equation. Thus, direct comparison of such BMDs to those for endpoints of other types of toxicity would not be possible. Nevertheless, within the context of EAC screening, a specific ED_{100x} value with confidence limits might be useful for prioritizing additional testing.

Evaluation of protocols for studying uterine responses:

Uterine responses have been used for evaluating activity of compounds and for research on estrogen-mediated processes. Variations in assay methods and the responses observed, which are briefly reviewed here, play a major role in the disparate dose response behaviors observed.

Studies in rodents have used two major animal models, immature animals prior to initiation of cycling and ovariectomized adult animals. Although the data in this paper

were restricted to studies with rats, mice have also been used extensively (Shelby *et al.*, 1996). Neonatal animals have been evaluated, but are used much less frequently (Sheehan *et al.*, 1981; Sheehan *et al.*, 1995).

In addition to ovariectomy status, there are a large number of variations in the methods used for the uterine response assays (Table 2). These variations fall into three areas - exposure regimens, the test animals, and responses observed – all of which affect or reflect both the pharmacokinetics of the chemical being studied and the pharmacodynamics of the response.

The influence of exposure regimen on the role of pharmacokinetics in the uterine response assays was demonstrated by induction of uterine cell proliferation with estriol following a single injection in oil, but not in saline (Clark and Markaverich, 1983). Pharmacokinetic differences, of the kind illustrated by dosing with E2 in saline or oil (Figure 6) (Jensen and Jacobson, 1962), likely account for the varied responses with estriol dosing. Oil dosing led to peak levels of E2 in uterus at 6 hours, a time at which receptor occupancy has been correlated with the cell proliferation response observed at 24 hours (Jensen *et al.*, 1966; Anderson *et al.*, 1972). Peak levels with saline as vehicle occurred much earlier. The protocol of O'Connor et al. (1996) used multiple daily doses to minimize the chances that rapidly cleared agonists would be missed in the uterine assay. Silastic implants and other methods have been used experimentally to produce continuous exposures (Markaverich *et al.*, 1984; Sheehan *et al.*, 1984; Medlock *et al.*, 1991). None of these exposure methods mimic the sustained and increasing concentrations of E2 that occur during estrus cycling, though the prolonged release from silastic implants is somewhat more realistic in this regards.

The doses of E2 (given by s.c. injection in saline vehicle) that are frequently used in uterine response assays have a steeply decreasing blood time course (Figure 7). Much higher blood concentrations are achieved for short periods than those found in immature females or during the estrus cycle. For example, during the estrus cycle blood concentrations of E2 were reported to range between 6 pg/ml (0.022 nM) and 48 pg/ml (0.18 nM) producing a range of 8-fold during the cycle (Smith *et al.*, 1975). Serum E2 levels in immature females (19 - 25 days) show generally similar levels and variations due to circadian rhythms and developmental changes (Dohler and Wuttke, 1975; Dohler and Wuttke, 1976). Subcutaneous injection doses often range between 0.01 and 5 μg per rat (equivalent to 0.2 - 100 μg/kg for an immature rat of 50 g). Subcutaneous

injection of 3 μg/kg (0.1 μg /30 g rat) aqueous E2 gave blood levels ranging from 3 down to 0.4 nM over 2 hours (Jensen and Jacobson, 1962), while i.v. injection of 2.5 μg/kg (0.25 μg/100 g rat) in water produced blood levels ranging from 13 down to 0.09 nM at 4 hours (Eisenfeld, 1967). Thus, intermediate doses by injection produce E2 blood levels that exceed, then approximate, those during the estrus cycle, with a profile over time (decreasing hormone following a peak exposure) essentially opposite to that occurring during cycling (e.g. constant - estrus, metestrus - or increasing levels - diestrus, proestrus).

Effects of animal selection on pharmacodynamic differences are illustrated by comparing adult and juvenile rats and rats ovariectomized for varying lengths of time. In adult ovariectomized animals, cell proliferation occurs in the epithelial and stromal cells of the endometrium, while in immature juveniles it occurs in all uterine cell types (Murphy and Ghahary, 1990). In ovariectomized rats, the weight of the uterus drops off steadily over several weeks reflecting adaptation to the loss of endogenous estrogen (Sheehan *et al.*, 1984). These pharmacodynamic variations arise from genuine differences in estrogen responsiveness during the life of the animal and likely affect the dose response characteristics of the different assay protocols.

Uterine responses:

Assays for estrogen agonist activity have largely focused on readily measured endpoints, water retention and tissue wet weight (Reel *et al.*, 1996). A few other endpoints, such as increased epithelial cell height have been included in some screening assays with juvenile and adult animals (Branham *et al.*, 1993; O'Connor *et al.*, 1996).

A much larger number of responses have been evaluated in mechanistic studies of the regulation of uterine responses in juvenile or adult animals by the estrogen receptor (Table 1). Activation of responses to estrogen agonists requires coordinated and sequential responses involving numerous genes (estimates range as high as several thousand DNA sites per cell nucleus involved in ER binding). Some of the differences in dose response observed for different endpoints likely reflect the underlying gene regulatory processes - for example, induction of single proteins as

compared to outcomes, such as increased epithelial cell height or uterine cell proliferation, that require altering groups of genes.

Classically, uterine responses were grouped temporally as early and late responses in relationship to a single dose of E2. The early responses include those required to recruit quiescent (G0) cells and prepare them (e.g. synthesis of ribosomes or c-fos mRNA and protein) for the subsequent cell proliferation response (Figure 8) (Musgrove and Sutherland, 1994; Altucci *et al.*, 1997). Other early responses, such as alterations in blood flow and vascular permeability for eosinophils or progesterone receptor induction, are cellular and tissue changes preparatory for events occurring following ovulation or implantation of a fertilized egg (Huet and Dey, 1987).

This empirical grouping is useful, particularly, due to the identification of short-acting estrogen agonists (Clark and Markaverich, 1983). These are compounds, such as estriol, which stimulate early events following a single aqueous dose, but not cell proliferation due to some combination of more rapid clearance and lower affinity for the estrogen receptor. Thus, it has been demonstrated that single doses of both E2 and estriol activate 'immediate-early' genes (e.g. c-fos, c-myc), but that additional estrogen-regulated cell cycle regulatory events are required to complete G1 and enter S phase (i.e. DNA synthesis, a "late" event) (Cosse-Mitchell *et al.*, 1988, Stancel *et al.*, 1994).

The ordering of events as early and late reflects the time following estrogen dosing and can be very different from that occurring *in vivo* during the estrus cycle. Increased blood flow, for example, is an "early" event occurring rapidly following estrogen injection, but it occurs late in the cycle, in proestrus. Only at that point are there high enough E2 blood and uterine concentrations to form sufficient ligand-receptor-DNA complexes to activate this response (Clark and Peck, 1979; Kerr *et al.*, 1992). Uterine water imbibition, an early event following single injections, has been suggested to be an important event late in the estrus cycle affecting implantation (Huet and Dey, 1987). Similarly, cell proliferation occurs during later times in the estrus cycle, diestrus and proestrus, when there are increasing E2 concentrations (Kaye *et al.*, 1972). The ordering of events leading to cell proliferation is likely to be largely the same regardless of continuous increasing or pulsatile exposures. However, the ordering of essentially independent events (e.g. increased blood flow, eosinophil infiltration, cell proliferation) may vary with the different exposures, their associated pharmacokinetics, and resulting occupancy of receptors and DNA sites for gene regulation.

While measuring wet weight is technically simple, the underlying processes are not, so different compounds do not all produce identical effects with identical time courses. Uterine wet weight at 6 hours is considered a measure of water retention while at 24 hours it is typically considered an indicator of cell proliferation. However, these characterizations were derived from the time course of injected E2 and they do not necessarily hold for other compounds. For example, coumestrol causes increases in uterine wet weight 24 hours following one or two daily doses (Markaverich *et al.*, 1995; Odum *et al.*, 1997) but no increase in DNA content was observed following the single dose (Markaverich *et al.*, 1995) (B. Markaverich confirmed that the labels in their Figure 3 were reversed - no increase in DNA content was observed). Whether this indicates that coumestrol is a short acting estrogen like estriol or has gene-specific agonist properties has not been adequately addressed. In addition, the time course can be dose dependent so that the times associated with maximum effects may vary (Reel *et al.*, 1996). These pharmacokinetic and pharmacodynamic factors will influence the apparent relative potency of different compounds, and will lead to some differences with variations in assay protocols.

Dose Response Analysis for Uterine Responses:

Empirical analyses based upon administered dose provide a rapid method for evaluating the relationship between dose and response and will continue to be widely used. As demonstrated by the analyses presented here, they provide essentially no basis for understanding the dose response characteristics of either the intact cycling system or the artificial screening systems (e.g. ovariectomized females). As long as uterine response assays are used strictly a screening tool for identifying and prioritizing which compounds should undergo more comprehensive testing for their potential to cause endocrine disruption, the empirical analysis is adequate.

While uterine responses are not adverse endpoints that might be used in human health risk assessment, they have been useful for evaluating issues that would arise for adverse receptor-mediated endocrine endpoints. For such effects, the empirical analysis will be of limited utility and further work would be required to describe the factors creating the dose response behavior. Biological responses result from a series of steps that begin with exposure to a compound, followed by its absorption, distribution,

metabolism, and elimination, i.e. pharmacokinetics, which are then followed by the series of biological events (i.e. pharmacodynamics) leading to the final response. By including these intermediate steps in dose response analysis it becomes possible to understand which factors control the extent of response and to rationalize and integrate responses observed under different conditions (i.e. different dosing regimens or different compounds) (Clewell and Andersen, 1987; Conolly *et al.*, 1988).

Such a biologically based analysis would provide a context for interpreting the dose response relationships observed in the various uterine response assays. For example, the injection route typically used in not terribly relevant for human health risk assessment absent appropriate dose-route extrapolations. It is striking that serum E2 concentrations change by only about 4- to 8-fold between maximum and minimum in cycling rats. Yet, many, though not all, of the uterine assays showed changes in response over much wider ranges raising questions about their relationship with normal physiology in adult cycling rats.

A fundamental question concerning risk assessment for EACs is what effect incremental exposure to exogenous compounds will have on intact animals, i.e. animals with endogenous production of E2. To address this issue it is necessary to start by evaluating the effects of exposure to E2, because this characterizes the physiological system with which the EAC must interact. A physiologically-based pharmacokinetic model for E2 has been developed (Plowchalk, 1998) and provides a start on this process. This model can be extended to incorporate other exposure methods such as silastic implants and injection that have been widely used for uterine response assays. Data also needs to be collected under conditions that better mimic endogenous E2 production – for example, silastic implants produce increasing serum E2 during their first day of implantation (Sheehan *et al.*, 1984). The pioneering studies of Clark and coworkers sytematically varied E2 doses and times at which responses were determined (Clark and Peck, 1979). This approach was invaluable to establishing much of what is known about the relationship between hormone levels, receptor occupancy, and response. New studies that utilize the contemporary molecular biological tools in the context of E2 dose and time could provide substantial information to unravel the factors that quantitatively control the dose response behaviors of this system.

Conclusions:

This analysis highlights several key decisions that need to be made when considering the use and widespread application of the uterine assay for screening potential EACs. The empirical dose response modeling with the Hill equation demonstrated differences among estimates of parameters that reflect, in part, widely disparate experimental protocols. Standardization of the assay would reduce the potential for such confounding and make comparisons among compounds more meaningful. The specific design suggestions offered above would improve the accuracy and precision of the empirical analysis and would help avoid pitfalls associated with peculiar model predictions (e.g., unreasonably high predictions of maximal response). This analysis identified several issues that will need to be resolved if the Hill equation is to be used for obtaining a BMD for use in risk assessment based upon adverse receptor-mediated endocrine endpoints. Attention must now be given to determining the definition of the BMD that is toxicologically meaningful and will most satisfactorily meet risk characterization needs. Finally, we described approaches to developing an understanding of the physiological processes controlling the extent of response in intact adult animals. To date, pharmacokinetic factors that influence response have been qualitatively identified (i.e. effects of oil versus saline vehicle), as have some early steps in the receptor-mediated response process. Development of a sound quantitative description of the uterine response to E2 would form a better basis for then evaluating the effects of exposures to other compounds with agonist or antagonist properties.

ACKNOWLEDGEMENT

This work was funded by the Chemical Manufacturers Association.

REFERENCES

Altucci, L., Addeo, R., Cicatiello, L., Germano, D., Pacilio, C., Battista, T., Cancemi, M., Petrizzi, V. B., Bresciani, F. and Weisz, A. (1997). Estrogen Induces Early and timed Activation of Cyclin-Dependent Kinases 4, 5, and 6 and Increases Cyclin Messenger Ribonucleic Acid Expression in Rat Uterus. *Endocrinology* **138**, 978-984.

Anderson, J. N., Clark, J. H. and Peck, E. J., Jr. (1972). The Relationship Between Nuclear Receptor-Estrogen Binding and Uterotrophic Responses. *Biochem. Biophys. Res. Commun.* **48**, 1460-1468.

Anderson, J. N., Peck, E. J., Jr. and Clark, J. H. (1973). Nuclear Receptor-Estrogen Complex: Relationship Between Concentration and Early Uterotrophic Responses. *Endocrinology* **92**, 1488-1495.

Anderson, J. N., Peck, E. J., Jr. and Clark, J. H. (1975). Estrogen-Induced Uterine Responses and Growth: Relationship to Receptor Estrogen Binding by Uterine Nuclei. *Endocrinology* **96**, 160-167.

Barlow, R. B. (1980). *Quantitative Aspects of Chemical Pharmacology.* University Park Press, Baltimore.

Barnes, D. G., Daston, G. P., Evans, J. S., Jarabek, A. M., Kaviock, R. J., Kimmel, C. A., Park, C. and Spitzer, H. L. (1995). Benchmark Dose Workshop: Criteria for Use of a Benchmark Dose to Estimate a Reference Dose. *Regul Toxicol Pharmacol* **21**, 296-306.

Branham, W. S., Zehr, D. R. and Sheehan, D. M. (1993). Differential Sensitivity of Rat Uterine Growth and Epithelium Hypertrophy to Estrogens and Antiestrogens. *Proc Soc Exp Biol Med* **203**, 297-303.

Clark, J. H. and Mani, S. K. (1994). Actions of Ovarian Steroid Hormones. In *The Physiology of Reproduction.* (Knobil, E. and Neill, J. D., Eds.), pp. 1011-1059., Raven Press, Ltd., New York.

Clark, J. H. and Markaverich, B. M. (1983). The Agonistic and Antagonistic Effects of Short Acting Estrogens: A Review. *Pharmacol Ther* **21**, 429-453.

Clark, J. H. and Peck, E. J., Jr. (1979). *Female Sex Steroids.* Springer-Verlag, New York.

Clewell, H. J., III and Andersen, M. E. (1987). Dose, Species, and Route Extrapolation Using Physiologically Based Pharmacokinetic Models. In *Pharmacokinetics in Risk Assessment: Drinking Water and Health* pp. 159-182., National Academy Press, Washington, DC.

Conolly, R. B., Reitz, R. H., Clewell, H. J. and Andersen, M. E. (1988). Pharmacokinetics, Biochemical Mechanism and Mutation Accumulation: A Comprehensive Model of Chemical Carcinogenesis. *Toxicol Lett* **43**, 189-200.

Crump, K. S. (1984). A New Method for Determining Allowable Daily Intakes. *Fundam. Appl. Toxicol.* **4**, 854-871.

Crump, K. S. (1995). Calculation of Benchmark Doses from Continuous Data. *Risk Anal* **15**, 79-89.

Dohler, K. D. and Wuttke, W. (1975). Changes with Age in Levels of Serum Gonadotropins, Prolactin and Gonadal Steroids in Prepubertal Male and Female Rats. *Endocrinology* **97**, 898-907.

Dohler, K. D. and Wuttke, W. (1976). Circadian Fluctuations of Serum Hormone Levels in Prepubertal Male and Female Rats. *Acta Endocrinol.* **83**, 269-279.

Eisenfeld, A. J. (1967). Computer Analysis of the Distribution of [^{3}H]Estradiol. *Biochim. Biophys. Acta* **136**, 498-507.

EPA (1995). *The Use of the Benchmark Dose Approach in Health Risk Assessment.* US Environmental Protection Agency, EPA/630/R-94/007, Washington, DC

EPA (1996). *Proposed Guidelines for Carcinogen Risk Assessment.* Office of Research and Development, EPA 600-P-92-003C, Washington, DC

Gaylor, D. W. and Slikker, W., Jr. (1990). Risk Assessment for Neurotoxic Effects. *NeuroToxicology* **11**, 211-218.

Grunert, G., Porcia, M. and Tcernitchin, A. N. (1986). Differential Potency of Oestradiol-17b and Diethylstilboestrol on Separate Groups of Responses in the Rat Uterus. *J. Endocrinol.* **110**, 103-114.

Huet, Y. M. and Dey, S. K. (1987). Role of Early and Late Oestrogenic Effects on Implantation in the Mouse. *J. Reprod. Fertil.* **81**, 453-458.

Jensen, E. v. and Jacobson, H. I. (1962). Basic Guides to the Mechanism of Estrogen Action. *Recent Prog Horm Res* **18**, 387-414.

Jensen, E. V., Jacobson, H. I., Flesher, J. W., Saha, N. N., Gupta, G. N., Smith, S., Colucci, V., Shiplacoff, D., Neumann, H. G., DeSombre, E. R. and Jungblut, P. W. (1966). Estrogen Receptors in Target Tissues. In *Steroid Dynamics*. (Pincus, G., Nakao, T. and Tait, J. F., Eds.), pp. 133-157., Academic Press, New York.

Kavlock, R. J., Allen, B. C., Faustman, E. M. and Kimmel, C. A. (1995). Dose-Response Assessments for Developmental Toxicity IV. Benchmark Doses for Fetal Weight Changes. *Fundam. Appl. Toxicol.* **26**, 211-222.

Kaye, A. M., Sheratzky, D. and Lindner, H. R. (1972). Kinetics of DNA Synthesis in Immature Rat Uterus: Age Dependence and Estradiol Stimulation. *Biochim. Biophys. Acta* **261**, 475-486.

Kerr, M. B., Marshall, K. and Senior, J. (1992). A Comparison of the Uterotrophic Response in Cyclic and Ovariectomized Normotensive and Spontaneously Hypertensive Rats. *J. Endocrinol.* **135**, 263-269.

Kneifel, M. A., Leytus, S. P., Fletcher, E., Weber, T., Mangel, W. F. and Katzenellenbogen, B. S. (1982). Uterine Plasminogen Activator Activity: Modulation by Steroid Hormones. *Endocrinology* **111**, 493-499

Lan, N. C. and Katzenellenbogen, B. S. (1976). Temporal Relationships Between Hormone Receptor Binding and Biological Responses in the Uterus: Studies with Short- and Long-Acting Derivatives of Estriol. *Endocrinology* **98**, 220-227.

Lobo, R. A. and Cassidenti, D. L. (1992). Pharmacokinetics of Oral 17 β-Estradiol. *J. Reprod. Med.* **37**, 77-84.

Longcope, C., Gorbach, S., Goldin, B., Woods, M., Dwyer, J. and Warram, J. (1985). The Metabolism of Estradiol: Oral Compared to Intravenous Administration. *J. Steroid Biochem.* **23**, 1065-1070.

Loose-Mitchell, D. S., Chiappetta, C. and Stancel, G. M. (1988). Estrogen Regulation of *c-fos* Messenger Ribonucleic Acid. *Mol. Endocrinol.* **2**, 946-951.

Markaverich, B. M., Roberts, R. R., Alejandro, M. and Clark, J. H. (1984). The Effect of Low Dose Continuous Exposure to Estradiol on the Estrogen Receptor (Type I) and Nuclear Type II Sites. *Endocrinology* **114**, 814-820.

Markaverich, B. M., Webb, B., Densmore, C. L. and Gregory, R. R. (1995). Effects of Coumestrol on Estrogen Receptor Function and Uterine Growth in Ovariectomized Rats. *Environ. Health Perspect.* **103**, 574-581.

Medlock, K. L., Lyttle, C. R., Kelepouris, N., Newman, E. D. and Sheehan, D. M. (1991). Estradiol Down-Regulation of the Rat Uterine Estrogen Receptor. *Proc Soc Exp Biol Med* **196**, 293-300.

Murphy, L. J. and Ghahary, A. (1990). Uterine Insulin-Like Growth Factor-1: Regulation of Expression and Its Role in Estrogen-Induced Uterine Proliferation. *Endocr. Rev.* **11**, 443-453.

Murrell, J. A., Portier, C. J. and Morris, R. W. (1998). Characterizing Dose-Response I: Critical Assessment of the Benchmark Dose Concept. *Risk Anal* **18**, 13-26.

Musgrove, E. A. and Sutherland, R. L. (1994). Cell Cycle Control by Steroid Hormones. *Cancer Biol.* **5**, 381-389.

O'Connor, J. C., Cook, J. C., Craven, S. C., Van Pelt, C. S. and Obourn, J. D. (1996). An *In Vivo* Battery for Identifying Endocrine Modulators That Are Estrogenic or Dopamine Regulators. *Fundam. Appl. Toxicol.* **33**, 182-195.

Odum, J., Lefevre, P. A., Tittensor, S., Paton, D., Routledge, E. J., Beresford, N. A., Sumpter, J. P. and Ashby, J. (1997). The Rodent Uterotrophic Assay: Critical Protocol Features, Studies with Nonyl Phenols, and Comparison with a Yeast Estrogenicity Assay. *Regul Toxicol Pharmacol* **25**, 176-188

Plowchalk, D. R. (1998). Physiologically-Based Modeling of Estradiol Pharmacokinetics in Rats and Humans: A Tool for Studying the Dose-Response of Estradiol. *Toxicol. Sci.* **42**, Abstract # 1180.

Reel, J. R., Lamb, J. C., IV and Neal, B. H. (1996). Survey and Assessment of Mammalian Estrogen Biological Assays for Hazard Characterization. *Fundam. Appl. Toxicol.* **34**, 288-305.

Sheehan, D. M., Branham, W. S., Guiterrez-Cernosek, R. and Cernosek, S. F., Jr. (1984). Effects of Continuous Estradiol Administration by Polydimethylsiloxane and Paraffin Implants on Serum Hormone Levels and Uterine Responses. *J. Am. Coll. Toxicol.* **3**, 303-316.

Sheehan, D. M., Branham, W. S. and Medlock, K. L. (1995). An Estrogen Developmental Potency and Toxicity Assay for Environmental Estrogens. *Teratology* **51**, 196-197.

Sheehan, D. M., Branham, W. S., Medlock, K. L., Olson, M. E. and Zehr, D. R. (1981). Uterine Responses to Estradiol in the Neonatal Rat. *Endocrinology* **109**, 76-81.

Shelby, M. D., Newbold, R. R., Tully, D. B., K., C. and Davis, V. L. (1996). Assessing Environmental Chemicals for Estrogenicity Using a Combination of *In Vitro* and *In Vivo* Assays. *Environ. Health Perspect.* **104**, 1296-1300.

Smith, M. S., Freeman, M. E. and Neill, J. D. (1975). The Control of Progesterone Secretion During the Estrous Cycle and Early Pseudopregnancy in the Rat: Prolactin, Gonadotropin and Steroid Levels Associated with Rescue of the Corpus Luteum of Pseudopregnancy. *Endocrinology* **92**, 219-226.

Stancel, G. M., Boettger-Tong, H., Hyder, S. M., Kirkland, J. L. and Loose-Mitchell, D. S. (1994). Uterine *c-fos*: A Paradigm for Control of Gene Expression During Estrogen Stimulated Target Tissue Growth. In *Protooncogenes and Growth Factors in Steroid Hormone Induced Growth and Differentiation.* (Khan, S. A. and Stancel, G. M., Eds.), pp. 87-104., CRC Press, Boca Raton.

Zacharewski, T. (1997). *In Vitro* Bioassays for Assessing Estrogenic Substances. *Environ. Sci. Technol.* **31**, 613-623.

Table 1
Uterine Responses

Early	Late
Hyperemia (increased blood in uterus)	Several of the early response continue for extended periods
Eosinophil infiltration	Increased protein and RNA synthesis
Water retention	Cellular hypertrophy
Albumin accumulation	DNA synthesis and mitosis
Increased glucose metabolism	Cellular replication
Increased RNA polymerase activity	
Synthesis of enzymes	
Increased synthesis of histone and nonhistone proteins	
increased *c-fos*,*c-myc*, cyclin-dependent kinases, cyclins, insulin-like growth factor-1 mRNA and/or protein	
decreased *c-jun* mRNA	

References:
Clark and Peck, 1979; Murphy and Ghahary, 1990; Bigsby and Li, 1994; Altucci *et al.*, 1997

Table 2
Common Variations in Uterine Response Study Methods

Animals	
Species	Rats, Mice
Strains	Sprague-Dawley, Wistar, others
Age	Adult, juvenile, early postnatal
Animal status	Intact or ovariectomized; Time since surgery (1 day to 3 weeks)
Exposure	
Route	oral, subcutaneous injection, intraperitoneal injection, silastic implants
Dose matrix	water/saline, methyl cellulose, oil (olive, arachis)
Number of days dosed	1, 3, 4, 5
Number of doses per day	1, 3
Time to sacrifice (post dosing)	6, 24 hours, other

Table 3
Single Estradiol Exposure Studies

Reference	Test Animals	Dosing [a]	Time of Measurement (hours post-dosing)
Anderson *et al.*, 1972	Immature (21-23 days) Purdue-Wistar	SC (NaCl): 0.2, 1, 2, 8, 20, 50	24[b]
Anderson *et al.*, 1973	Immature (21-23 days) Purdue-Wistar	SC (NaCl): 0.2, 1, 2, 8, 20,50	3, 6[b]
Anderson *et al.*, 1975	Immature (21-23 days) Purdue-Wistar	SC (NaCl): 1.8, 18, 182	3
Gruhert *et al.*, 1986	Immature Sprague-Dawley	*i.v.*: 0.01, 0.1, 1, 10, 100, 300, 1000	6, 24
Kaye *et al.*, 1972	Immature (20 days) Wistar	*i.p.*: 0.0015, 0.015, 0.15, 1.5, 15, 150	24

Notes:

[a] Dosing Routes: subcutaneous (SC), Intravenous (*i.v.*), Intraperitoneal (*i.p.*). Dose values given in μg/kg. In many cases these are estimated, sometimes very grossly, based on information on dosage (μg) and body weights.

[b] Other time points included in paper were not suitable for modeling.

Table 4
Multiple Dosing Estradiol Studies

Reference	Test Animals	Dosing [a]	Time of Measurement (hours after last dosing)
Branham *et al.*, 1993	Immature (20-24 days) Sprague-Dawley	SC (oil) x 5: 0.017, 0.17, 1.7, 17, 167, 1667	2
Kneifel *et al.*, 1982	Immature (19 days) Sprague-Dawley	SC (oil) x 3: 0.6, 1.6, 2, 6, 20, 60, 200, 600	24
Lan and Katzenellenbogen, 1976	Immature (20-24 days) Holtzman	SC (NaCl) x 3: 1, 2, 6, 20, 60, 200	24
Odum *et al.*, 1997	Immature (21-22 days) Alpk: AP	SC (oil) x 3: 0.5, 1, 2, 10, 20, 40, 200, 400 Gavage (oil) x 3: 10, 20, 40, 100, 200, 400	24

Notes:

[a] Dose values given in µg/kg. In many cases, these are estimated based on information on dosage (µg) and body weights.

Table 5
Studies with Estriol

Reference	Test Animals	Dosing [a]	Time of Measurement (hours after last dosing)
Estriol			
Anderson *et al.*, 1975	Immature (21-23 days) Purdue-Wistar	single SC (NaCl): 1.8, 18, 182	3
Lan and Katzenellenbogen, 1976	Immature (20-24 days) Holtzman	SC (NaCl) x 3: 20, 60, 200, 600	24

Table 6
Hill Equation Parameters, *n* and ED_{50}, Fit to Uterine Weight Data Sets

Study Type	Reference [a]		Goodness of fit p-values	Parameter Estimates *n*	ED_{50}	Fig
Single E2 Exposures	Anderson *et al.*, 1972	(24)	.18	2.9	0.71	1A
	Grunert *et al.*, 1986	(24)	.35	1.3	0.29	
	Kaye *et al.*, 1972	(24)	.68	0.92	0.74	1B
	Anderson *et al.*, 1973	(6)	.35	3.4	0.49	3B
	Grunert *et al.*, 1986	(6)	.995	0.47	0.11	
	Anderson *et al.*, 1975	(3)	*	1.1	2.6	
	Anderson *et al.*, 1973	(3)	.46	1.6	2.2	3A
Multiple E2 Exposures	Kneifel *et al.*, 1982	(24)	.14	0.72	1.4	2A
	Lan and Katzenellenbogen, 1976	(24)	.09	0.48	2.7	2B
	Odum *et al.*, 1997	(24) - SC	.005	6.0	3.1	2C
	Odum *et al.*, 1997	(24) - gavage	.08	1.6	280	4
	Branham *et al.*, 1993	(2)	.77	0.42	1500	
Estriol Exposure	Anderson *et al.*, 1975	(3)	*	1.6	2.7	
	Lan and Katzenellenbogen, 1976	(24)	<.001	1.6	79000	5

Notes:

[a] In parentheses are the hours after last dosing at which time uterine weights were measured.

* No degrees of freedom for assessing fit. Visually, fit is perfect.

Figure 1: Uterine Wet Weight at 24 Hours Following a Single Injection Dose of E2. Responses at 24 hours are largely cell proliferation. **A.** The data of Anderson et al. 1972 were normalized to percent control response using 30.8 ± 1.4 mg for uterine weight in control animals. **B.** Data from Kaye et al. 1972 was normalized using a value of 22 ± 1.5 mg for uterine weight in control animals.

Figure 2: Uterine Wet Weight at 24 Hours Following Multiple Injection Doses of E2. Responses at 24 hours following multiple doses of E2 are largely cell proliferation. **A.** Data from Kneifel et al. 1982 **B.** Data from Lan and Katzenellenbogen, 1976 **C.** Data from Odum et al. 1997.

Figure 3: Uterine Wet Weight at 3 or 6 Hours Following a Single Injection Dose of E2. **A.** Response at 3 hours is predominantly water retention. **B.** 6 hour data is the maximum for water retention. Data from Anderson et al. 1973

Figure 4: Uterine Wet Weight at 24 Hour Following Three Oral Doses of E2. Data from Odum et al. 1997.

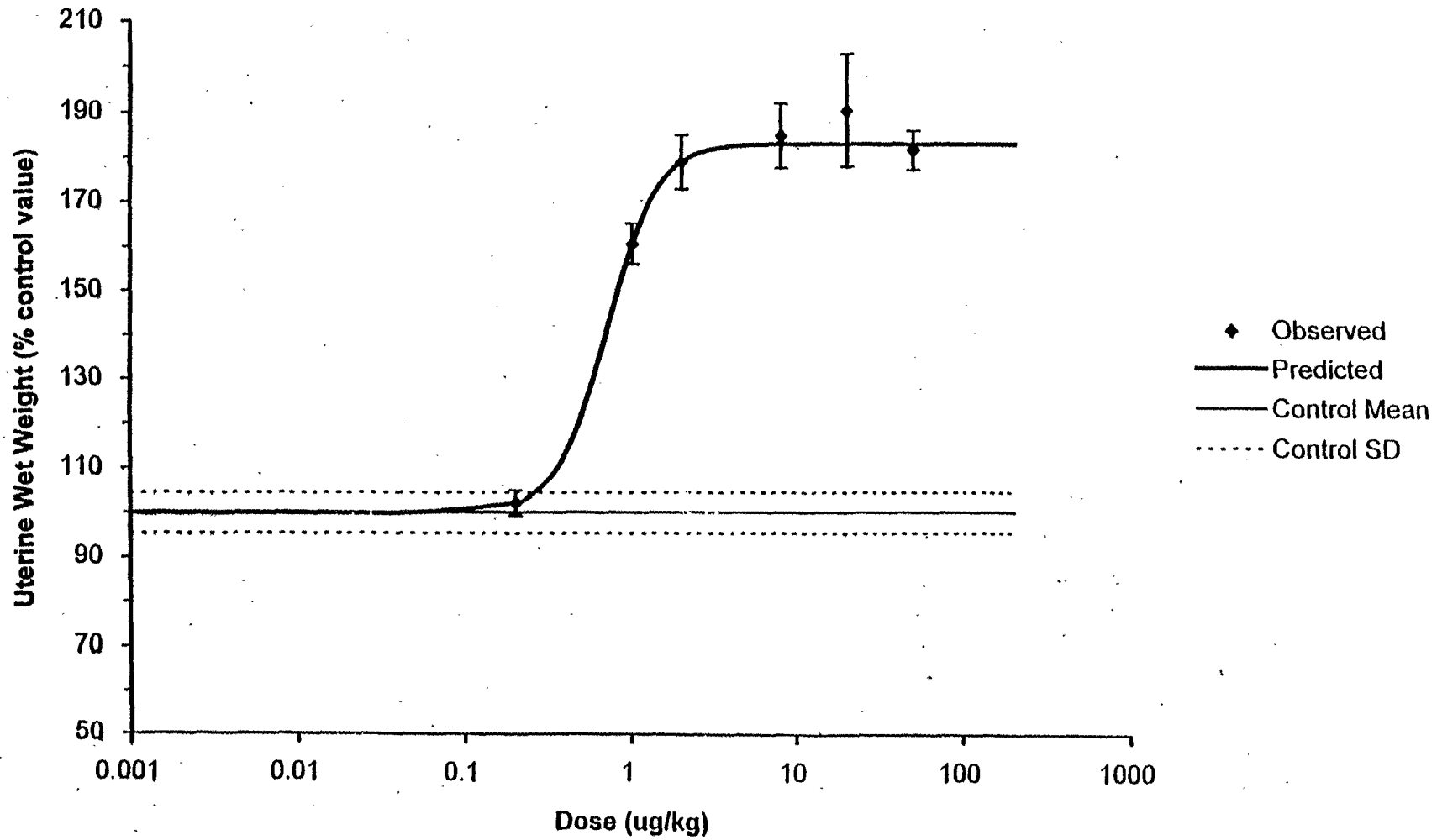
Figure 5: Uterine Wet Weight at 24 Hours Following a Single Injection Dose of Estriol. **A.** Fit of Hill equation to the data without constraint on maximum uterine weight. **B.** Constraining the value for Dm, maximum uterine weight, alters fit. Data from Lan and Katzenellenbogen 1976.

Figure 6: Uterine [^{3}H]E2 Following Dosing With E2 in Saline or Oil. Subcutaneous injection of E2 in saline resulted in rapid uptake prolonged through about 2 hours. Subcutaneous injection of E2 in oil resulted in slow prolonged uptake into the uterus. Intravenous injection of E2 in saline resulted in the rapid uterine uptake. Rats (23 days old) were injected with 0.1µg E2 (approximately 3µg/kg). Redrawn from Jensen 1962.

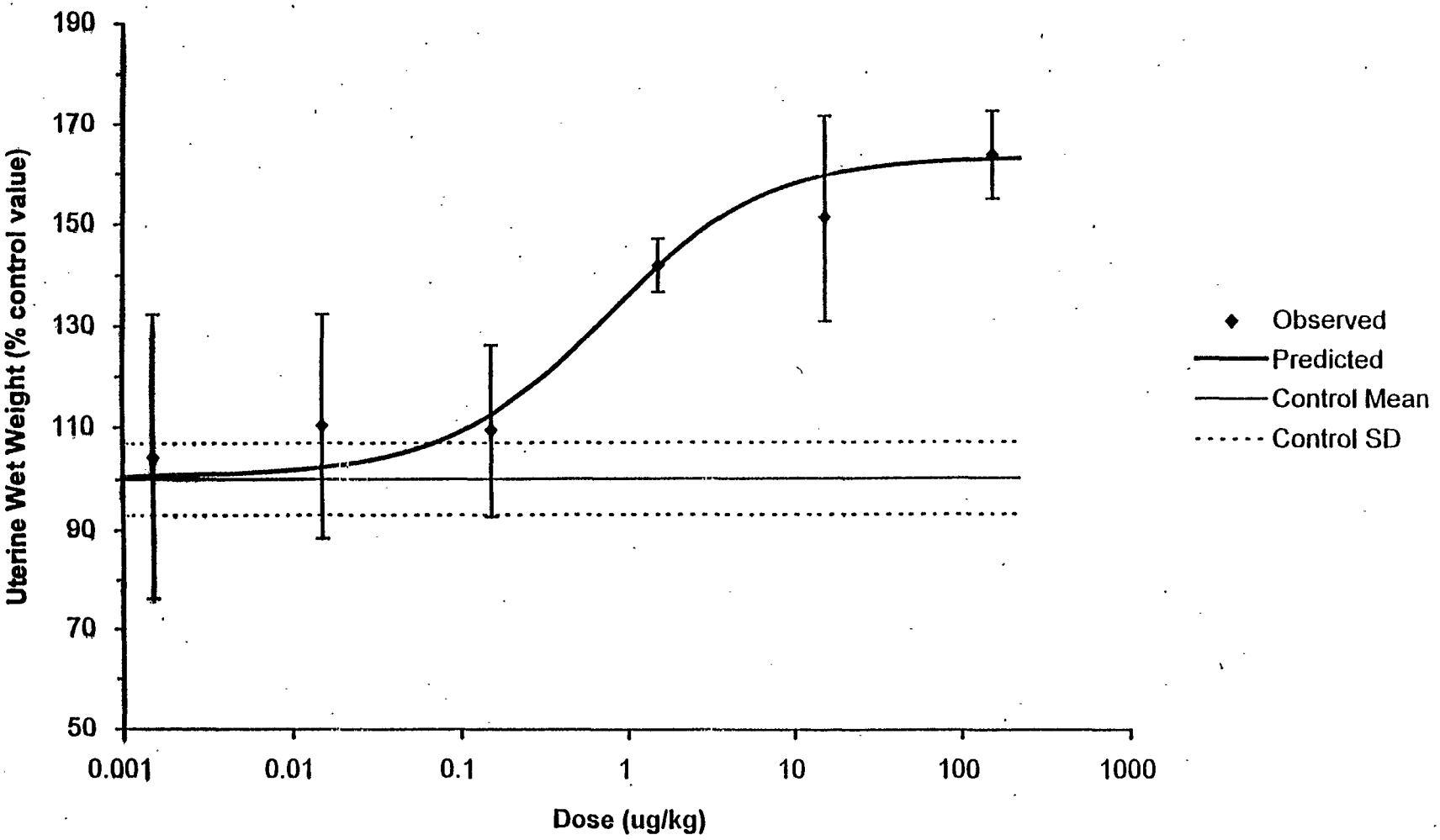
Figure 7: Blood and Uterine E2 or Estriol (E3) Equivalent Concentrations Following s.c. Injection in Saline. Hormone equivalents were calculated and concentrations estimated based upon reported radioactivity per gram dry tissue following dosing with 0.1 µg hormone per rat (Jensen, 1966). Analysis of tissue radioactivity at 2 hr found the uterine radioactivity to be exclusively parent hormone. Radioactivity in blood was predominantly water soluble conjugates (56% and 61% following E2 or E3 dosing, respectively). The ether soluble fraction (14% and 9% following E2 or E3 dosing, respectively) was distributed among estrone, E2, and E3 following E2 dosing (data not presented for E3).

Figure 8: Uterine E2 Exposure and Response. E2 is taken up by the uterus and binds to the estrogen receptor (R). Dimer of the E2R complex binds with estrogen response elements (EREs) in DNA and accessory proteins. Several responses occurring soon after s.c. injection dosing are illustrated - water retention, induction of progesterone receptor, and induction of proteins and RNA in preparation for cell cycling. Cell proliferation is a late event which occurs only in the presence of products of the early responses and continued occupancy of gene regulatory sites by E2R.

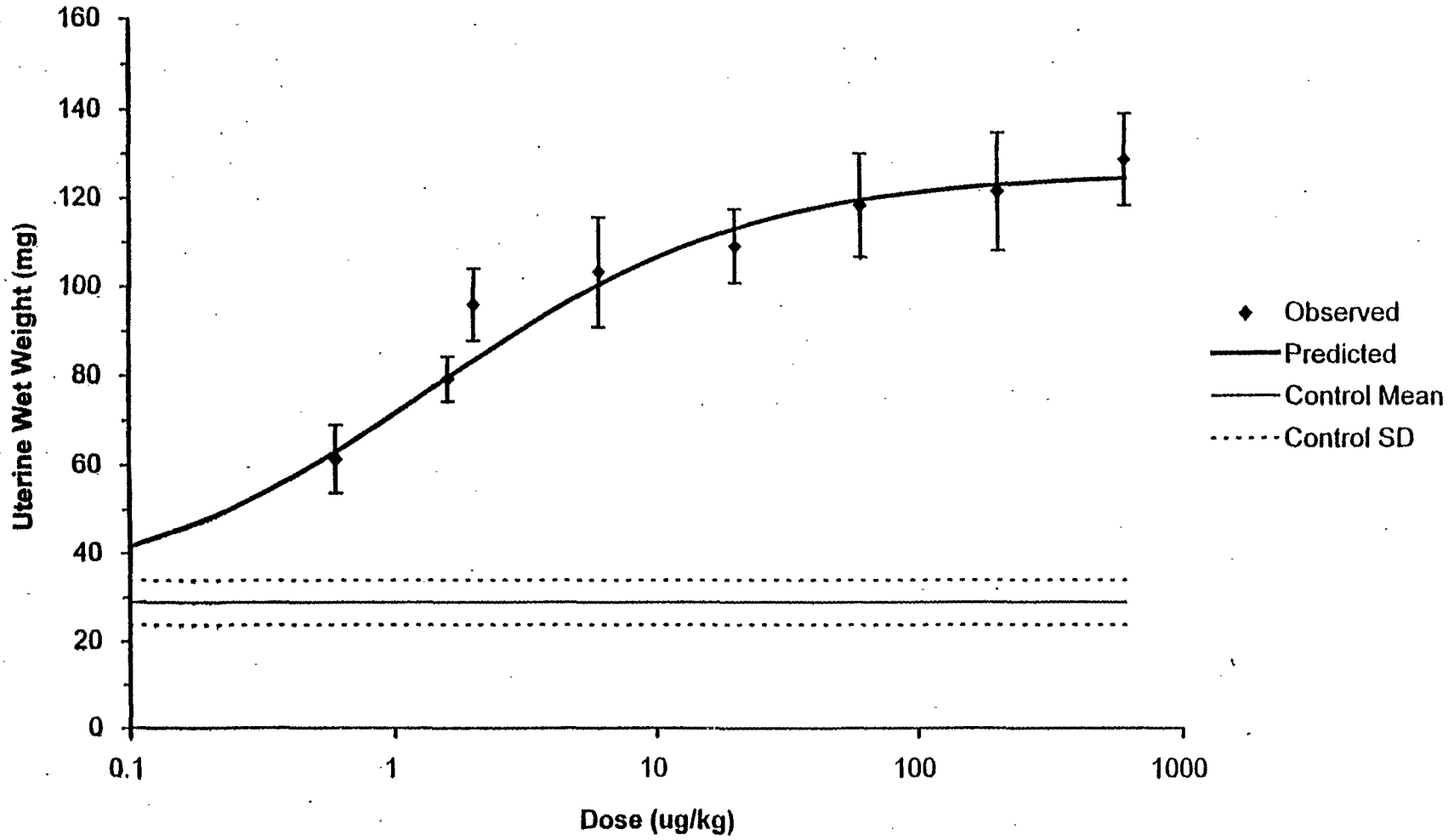
1A


210
190
170
150
130
110
90
70
50
Uterine Wet Weight (% control value)
0.001
0.01
0.1
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

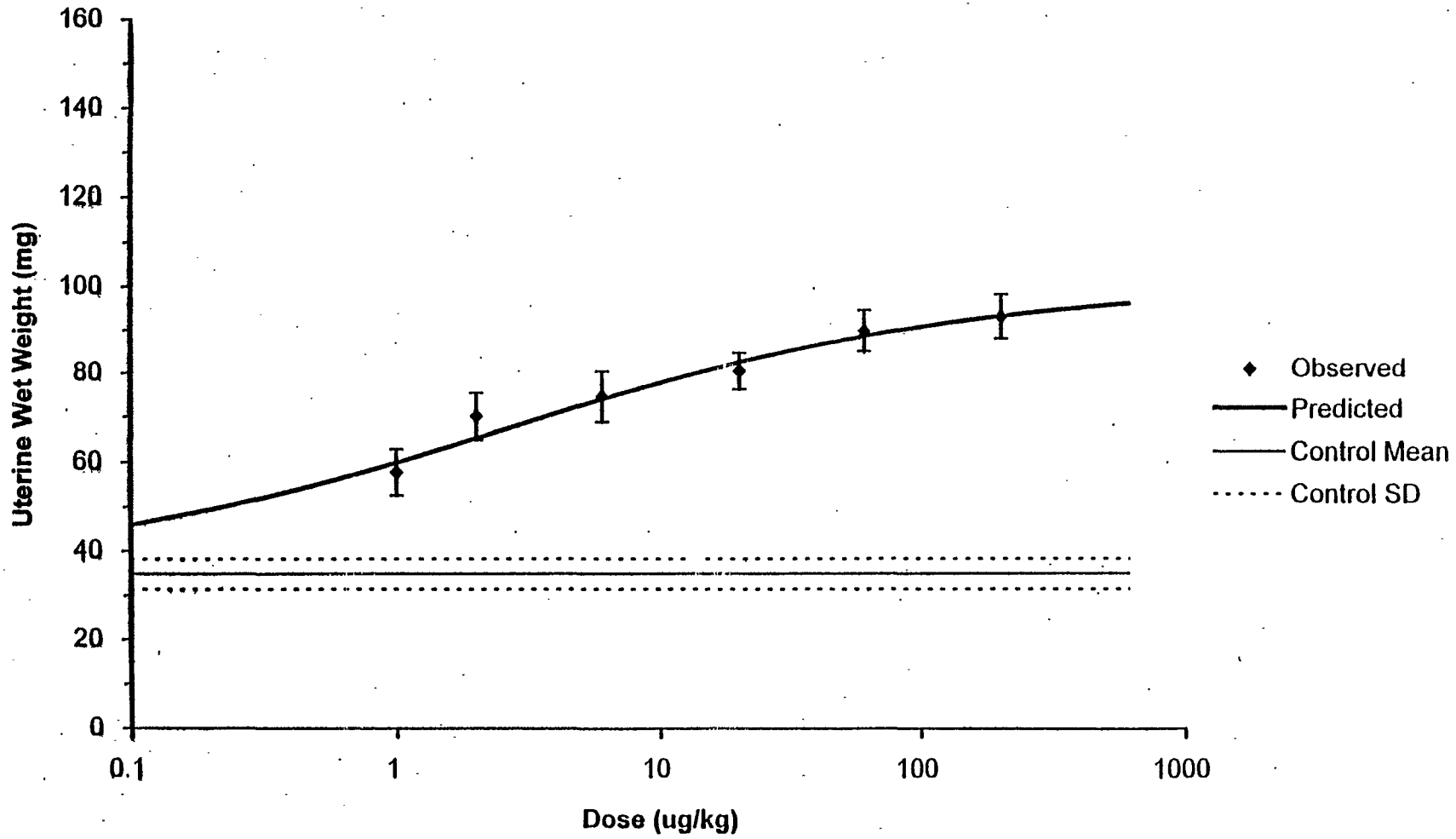
1B


190
170
150
130
110
90
70
50
Uterine Wet Weight (% control value)
0.001
0.01
0.1
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

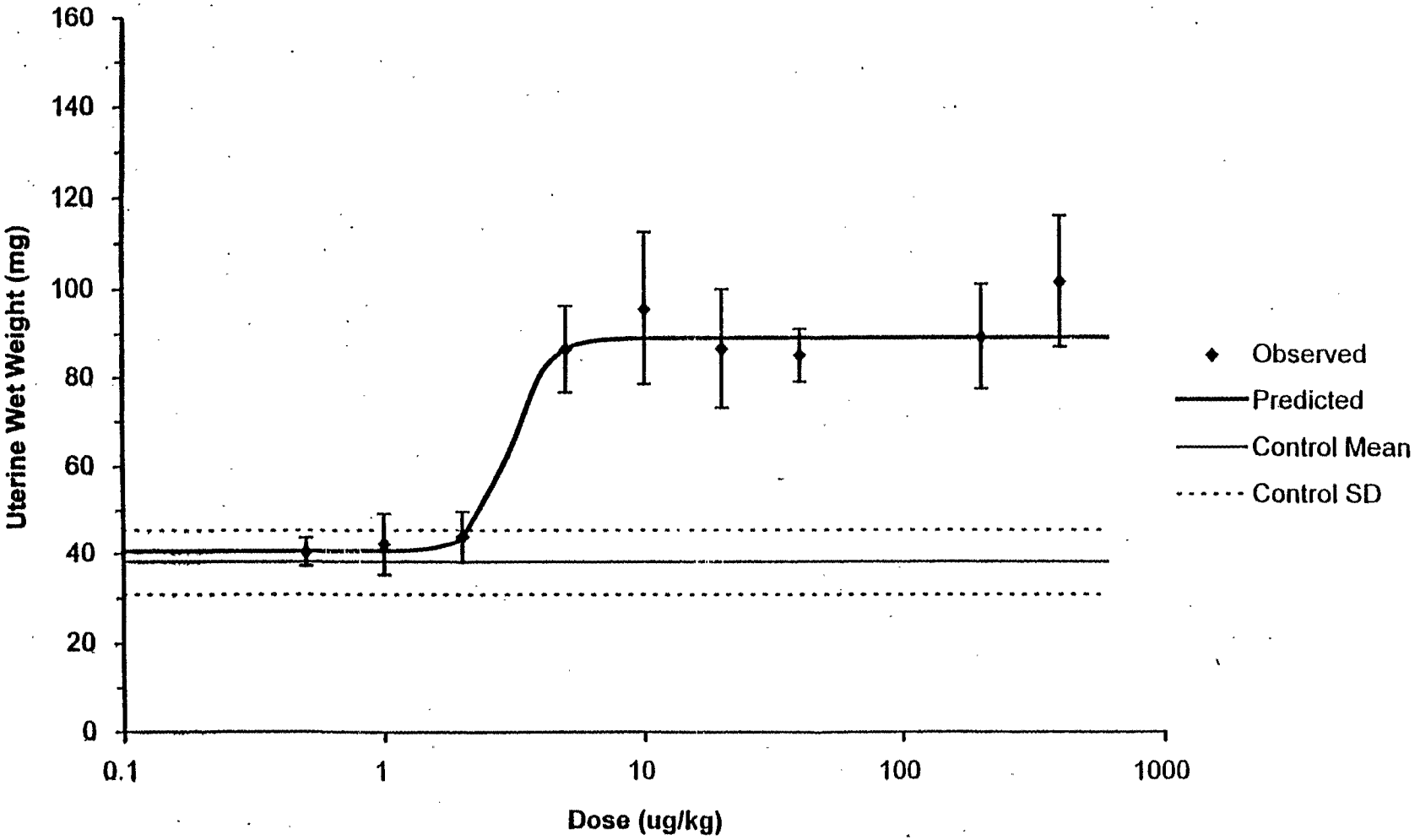
2A


160
140
120
100
80
60
40
20
0
Uterine Wet Weight (mg)
0.1
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

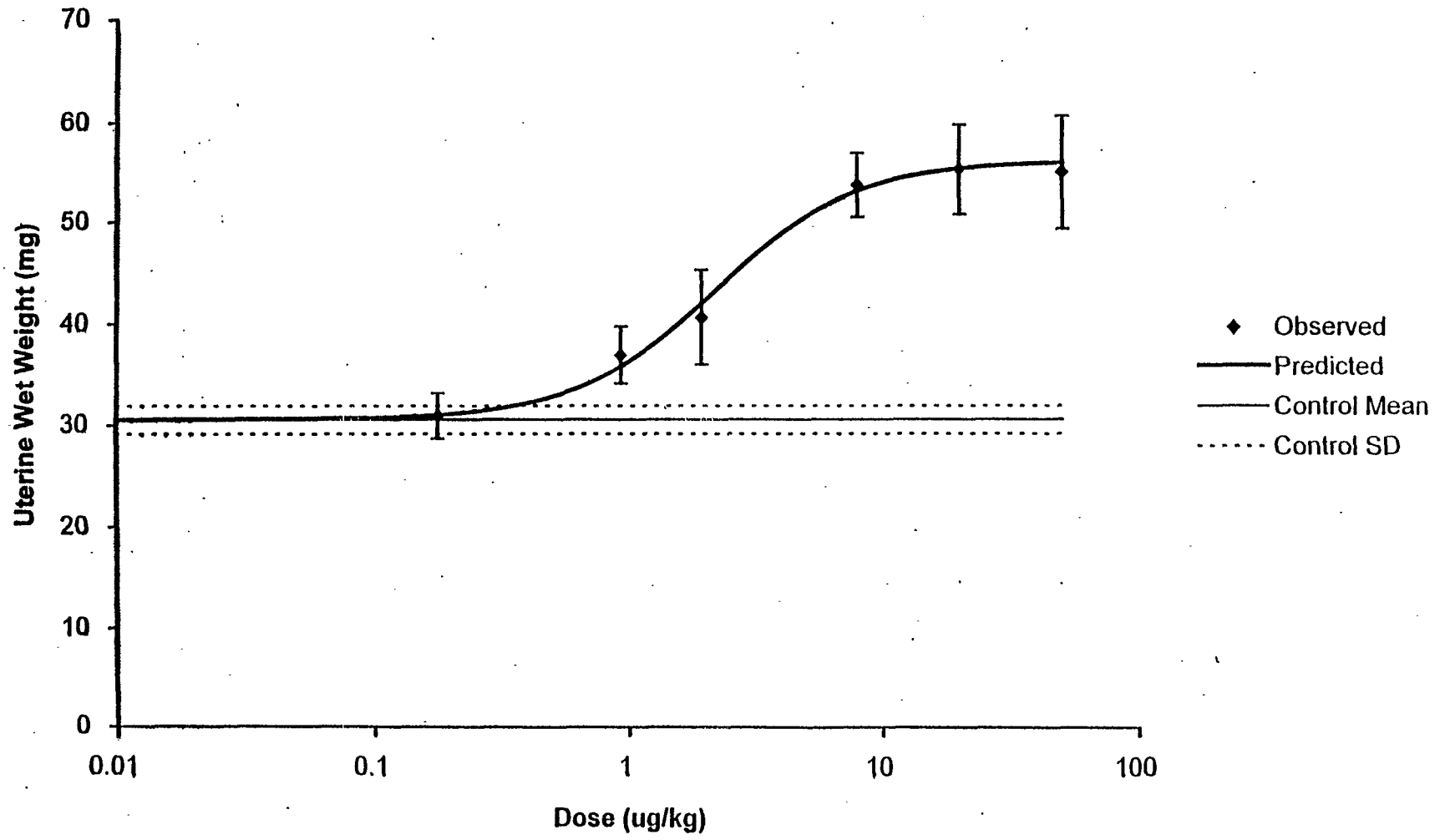
2B


160
140
120
100
80
60
40
20
0
Uterine Wet Weight (mg)
0.1
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

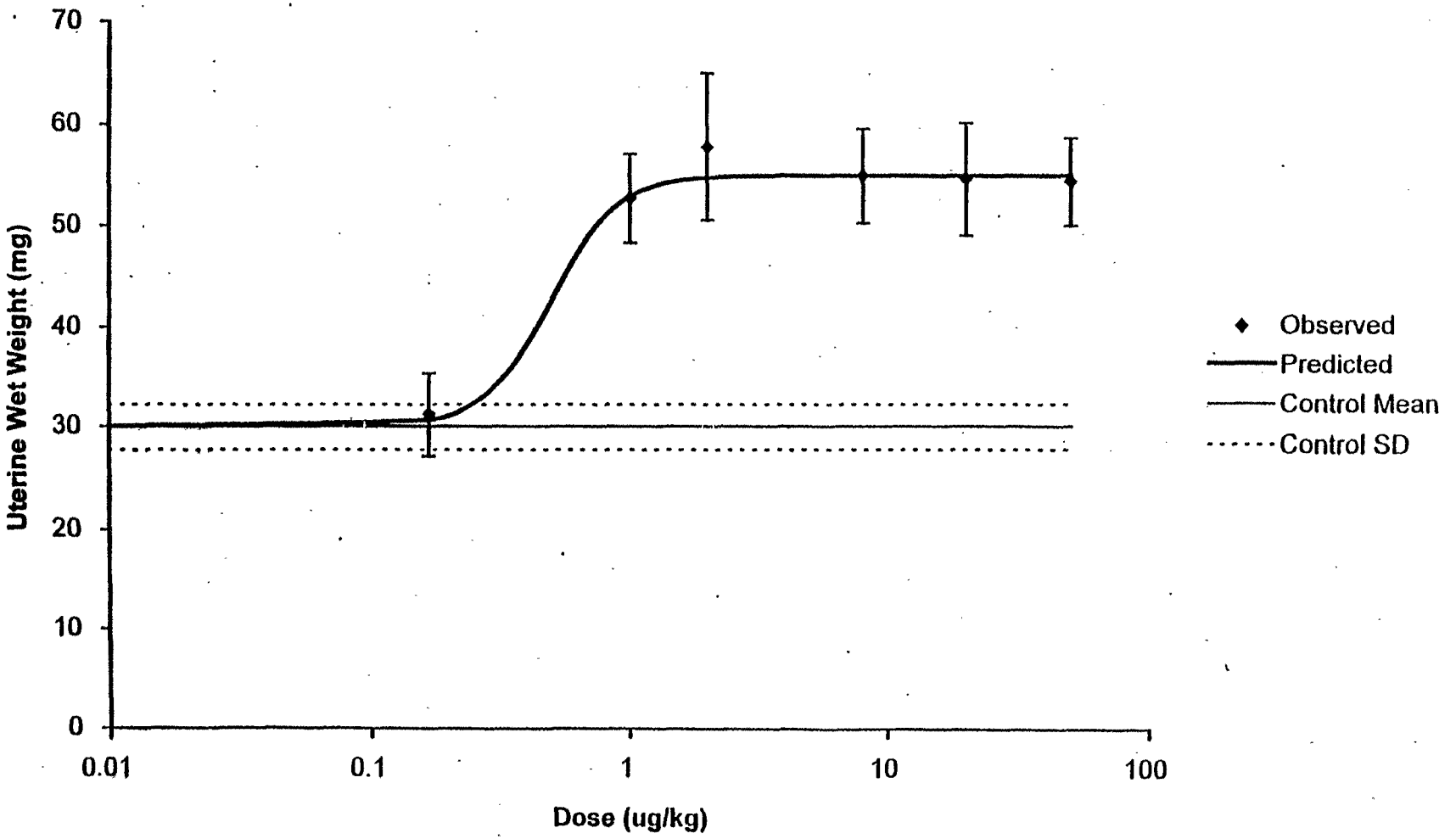
2C

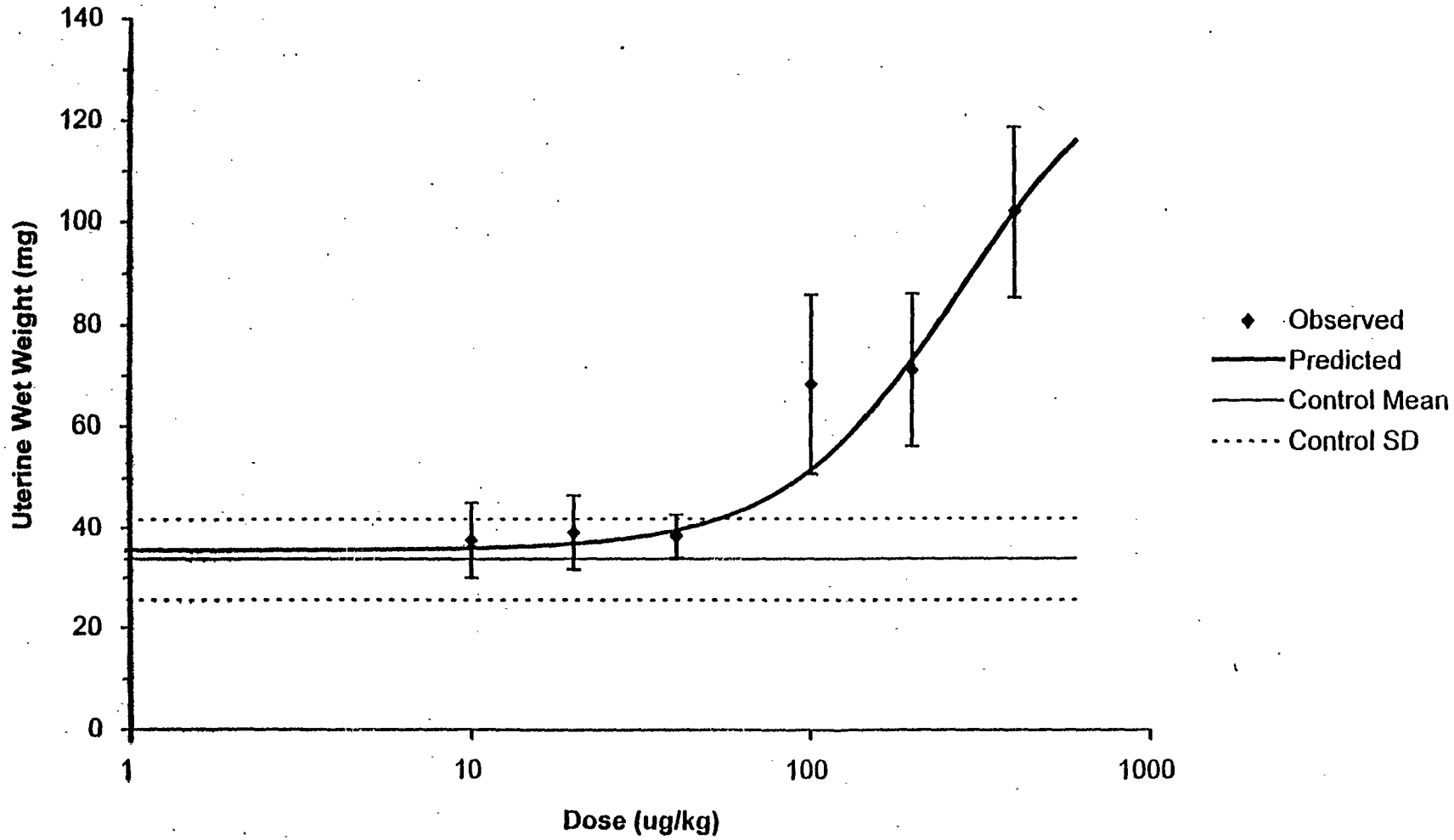

160
140
120
100
80
60
40
20
0
Uterine Wet Weight (mg)
0.1
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

3A

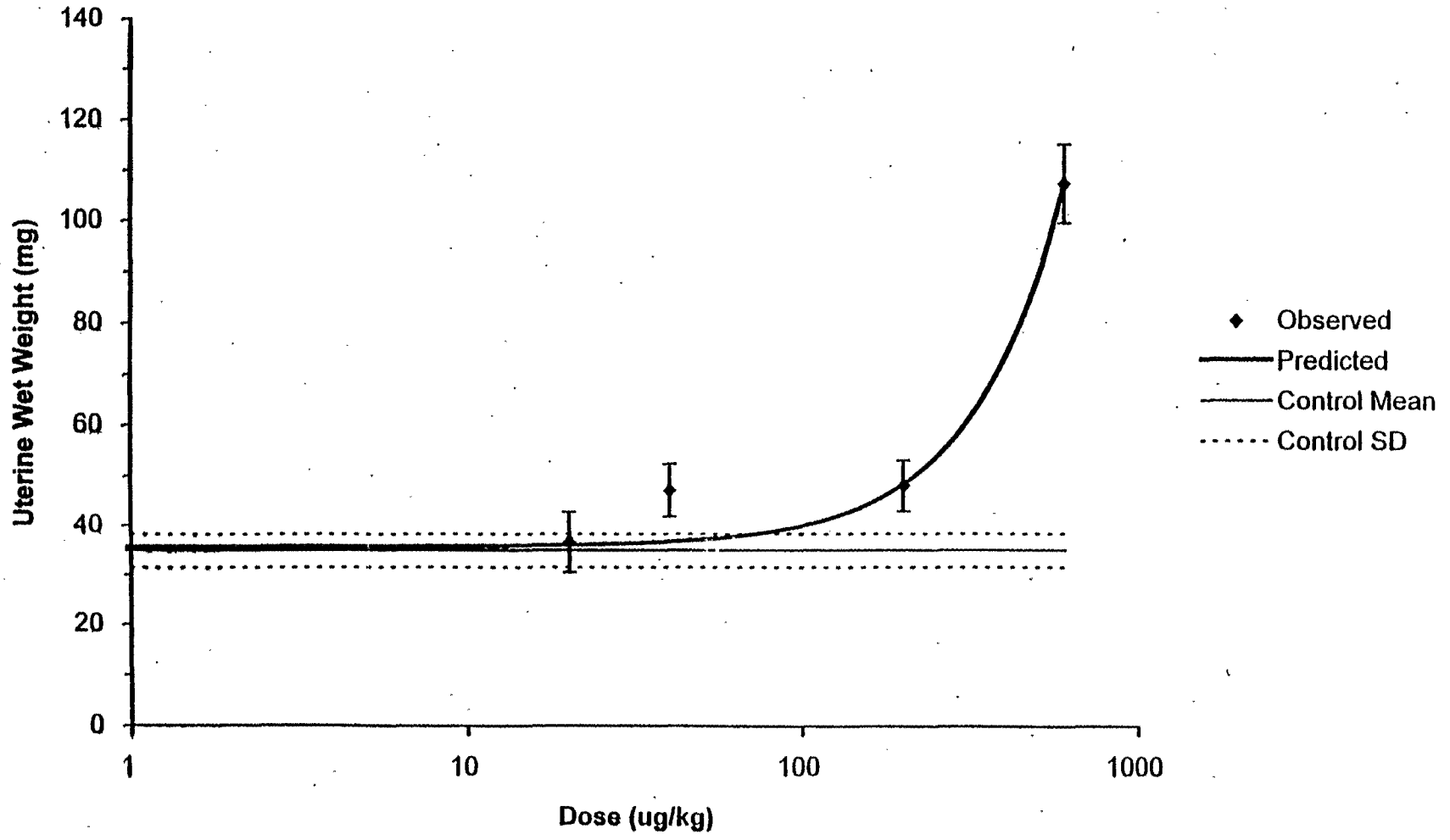

70
60
50
40
30
20
10
0
Uterine Wet Weight (mg)
0.01
0.1
1
10
100
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

3B

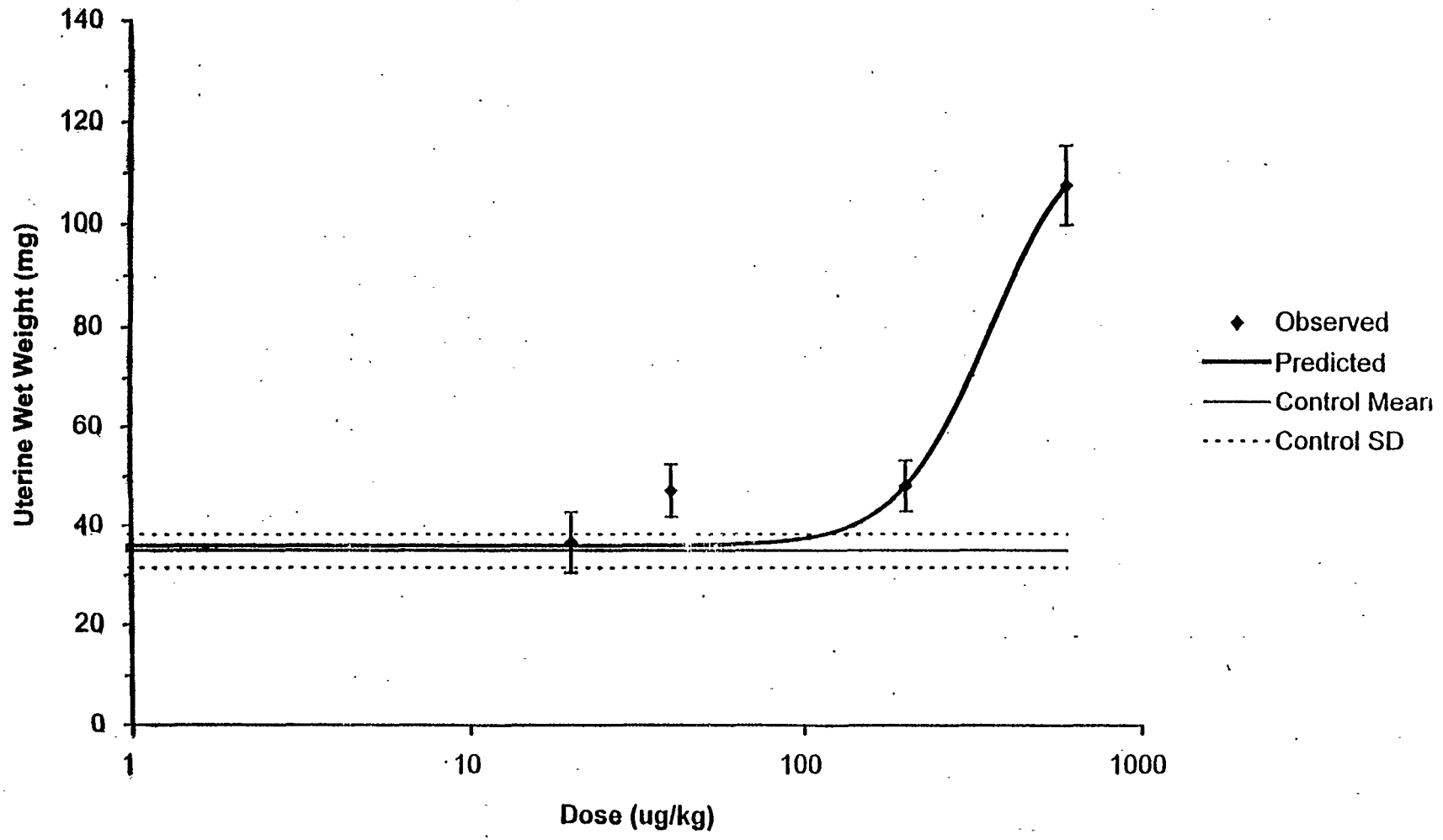

70
60
50
40
30
20
10
0
Uterine Wet Weight (mg)
0.01
0.1
1
10
100
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

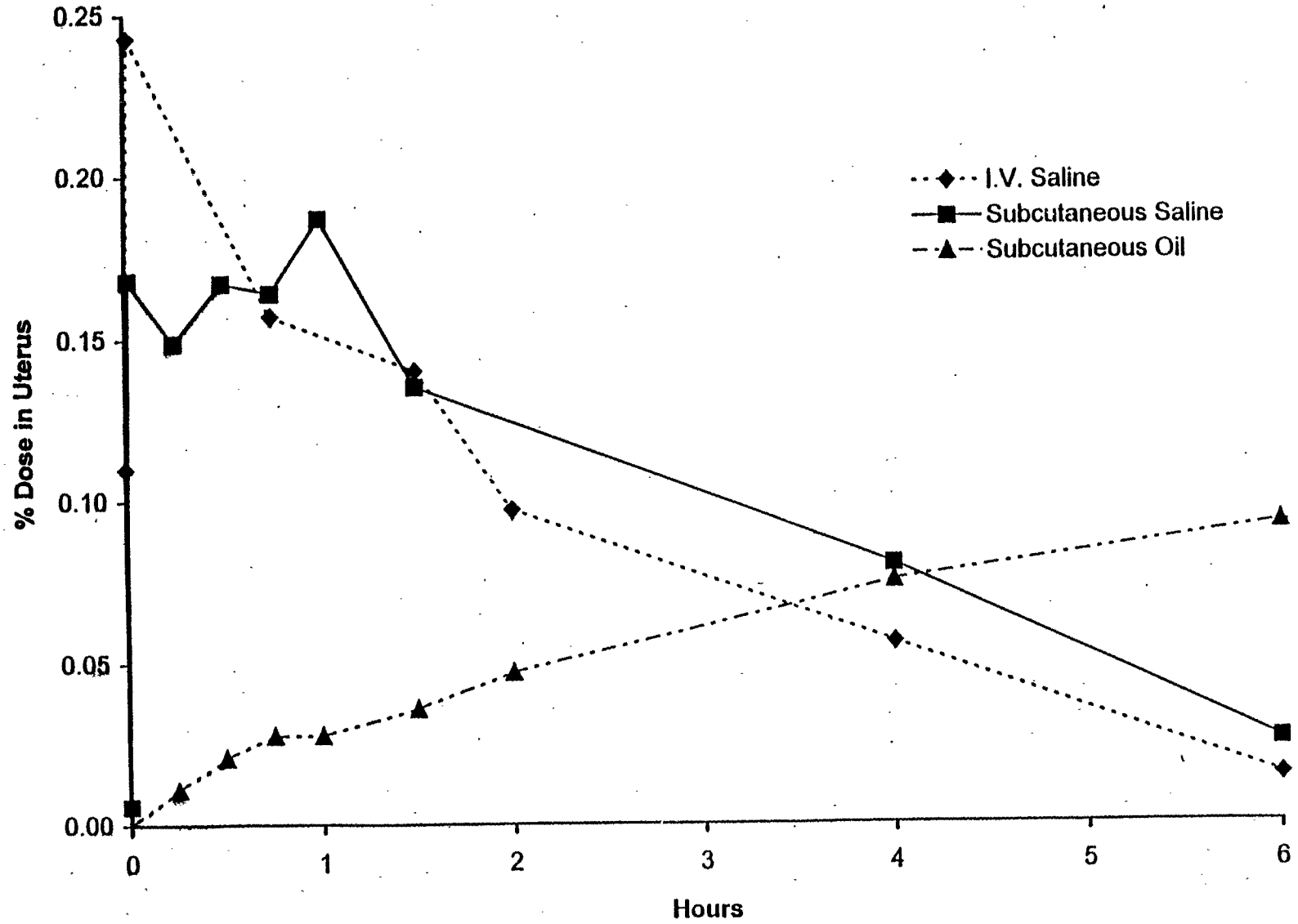

Uterine Wet Weight (mg)
140
120
100
80
60
40
20
0
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

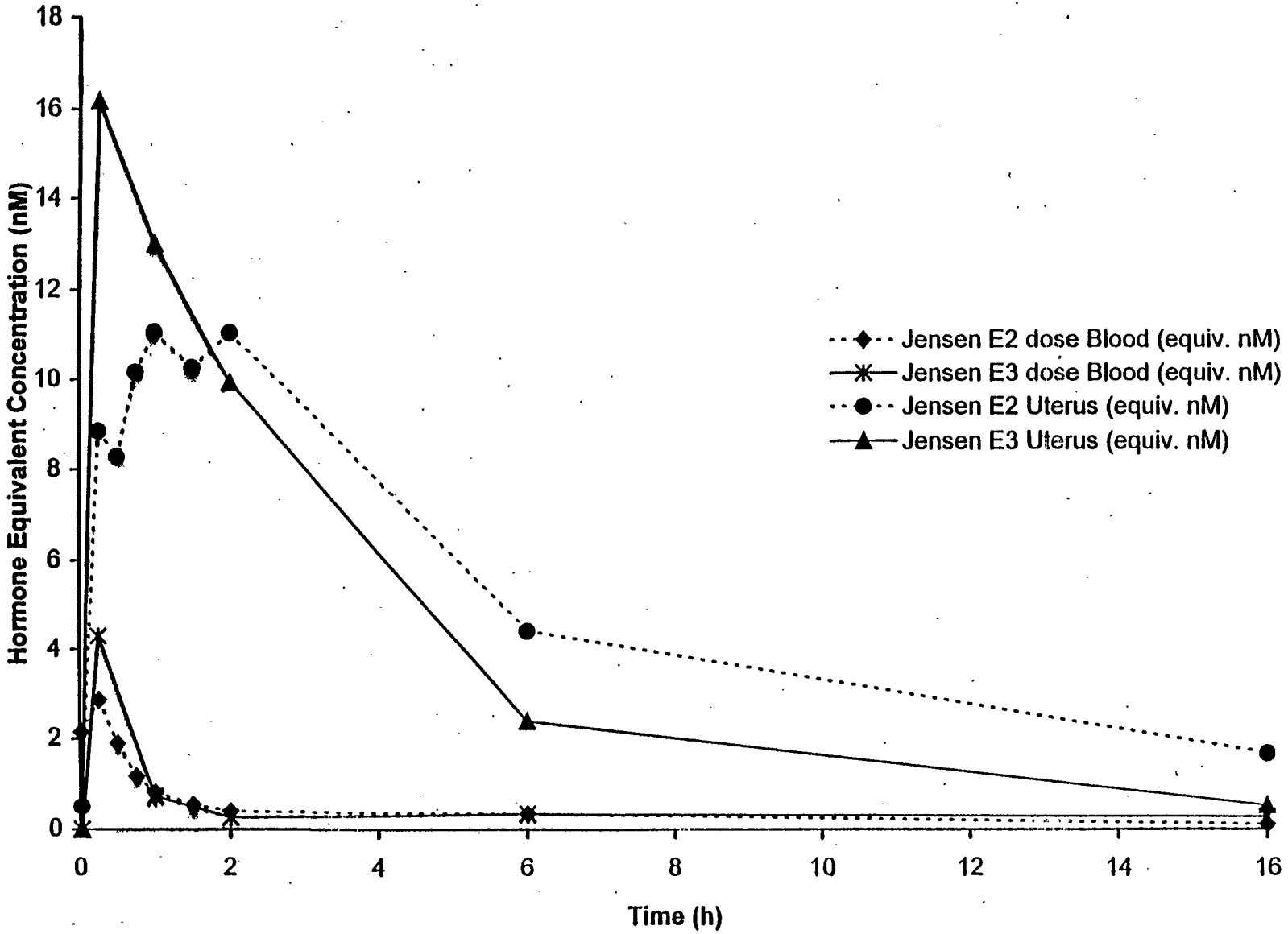
5A

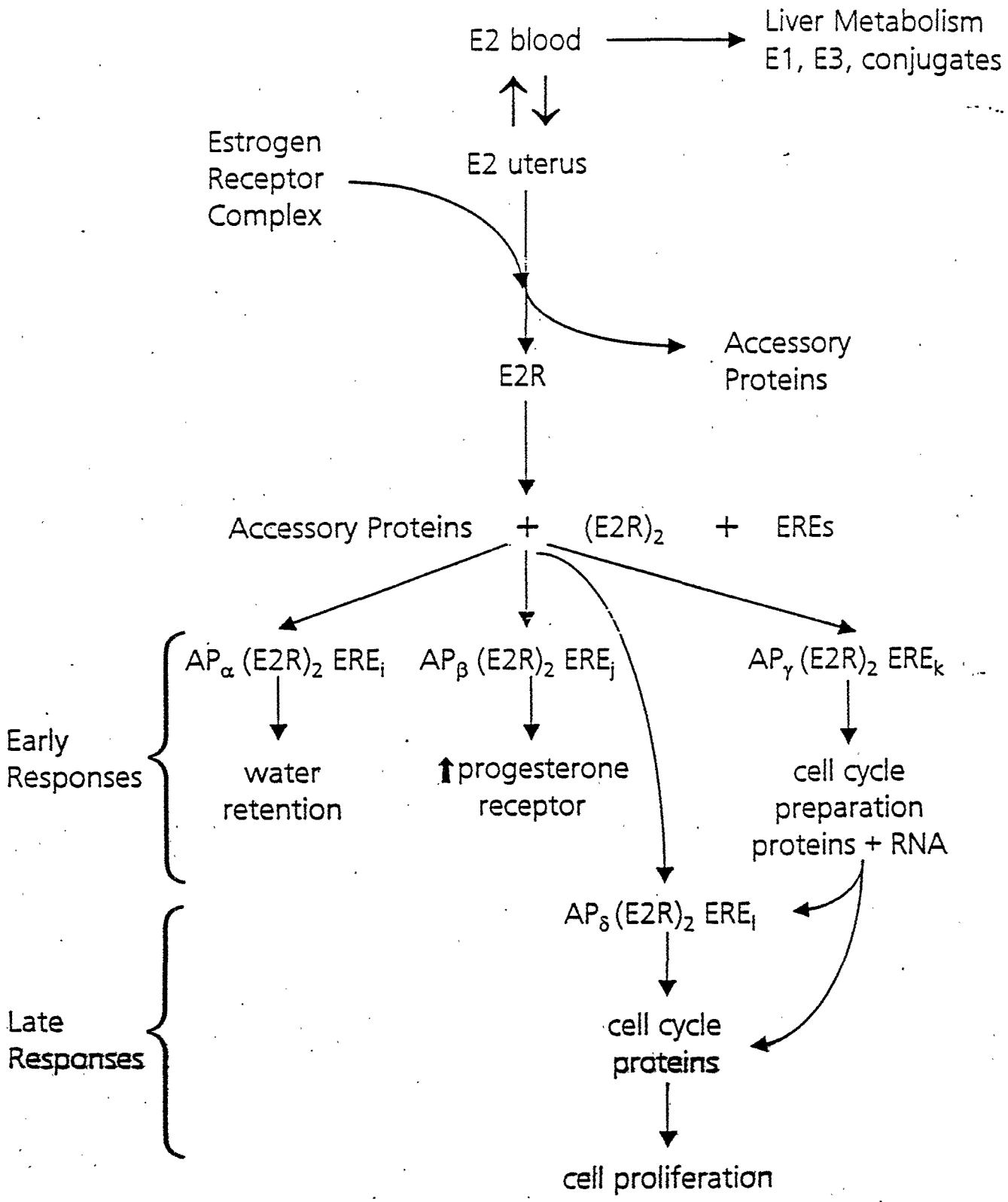

Uterine Wet Weight (mg)
140
120
100
80
60
40
20
0
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD

5B


Uterine Wet Weight (mg)
140
120
100
80
60
40
20
0
1
10
100
1000
Dose (ug/kg)
Observed
Predicted
Control Mean
Control SD


0.25
0.20
0.15
0.10
0.05
0.00
% Dose in Uterus
0
1
2
3
4
5
6
Hours
I.V. Saline
Subcutaneous Saline
Subcutaneous Oil


Hormone Equivalent Concentration (nM)
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
Time (h)
Jensen E2 dose Blood (equiv. nM)
Jensen E3 dose Blood (equiv. nM)
Jensen E2 Uterus (equiv. nM)
Jensen E3 Uterus (equiv. nM)


E2 blood
Liver Metabolism
E1, E3, conjugates
Estrogen
Receptor
Complex
E2 uterus
E2R
Accessory
Proteins
Accessory Proteins + (E2R)2 + EREs
APα (E2R)2 EREi
APβ (E2R)2 EREj
APγ (E2R)2 EREk
Early
Responses
water
retention
progesterone
receptor
cell cycle
preparation
proteins + RNA
APδ (E2R)2 EREl
Late
Responses
cell cycle
proteins
cell proliferation